# A MODIFICATION OF THE SHERMAN METHOD OF STUDYING THE MULTIPLE NATURE OF VITAMINS, WITH AN APPLICATION TO VITAMIN G

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Since the vitamin B complex was definitely shown to consist of at least two separate and distinct components, now referred to as vitamin B or $B_1$ and vitamin G or $B_2$, much evidence has been presented in the literature that each of these components is divisible into two or more entities. Reviewers of this work have often hesitated to assign to the evidence for some particular unknown factor any definitive significance, although admitting the belief that in all probability the old B complex consists of more than vitamins B and G.

The difficulties in the way of according full significance to such evidence are, first, that potent concentrates of the presumably unknown factors have so seldom been prepared, and, second, that the evidence of the existence of new factors does not consist of the production of characteristic symptoms of malnutrition by their removal from the diet, but merely of certain more or less well-defined and reproducible disturbances of growth, such as a 'plateau' in the curve of growth. It is perhaps too seldom realized that failure of growth is a non-specific phenomenon, in itself affording no indication of its etiology, and in particular that such failure may be the result of a quantitative dietary deficiency as well as of a qualitative deficiency. Also, the fact that a different amount

of a given vitamin concentrate is required to maintain weight or growth than to prevent or cure specific symptoms of vitamin deficiency is not valid evidence of the multiple nature of a vitamin, since Baumann, Riising and Steenbock ('34) have shown the same to be true of chemically pure carotene.

An ingenious method of testing the possible multiple nature of a given vitamin was applied by Sherman and Axtmayer ('27) to the old B complex, and later in the same laboratory by Stiebeling ('31–'32) and by Page ('34) to vitamin G($B_2$). The method consists of fractionating a selected good source of the vitamin under study by some likely chemical procedure. The two fractions are then tested separately and in equivalent amounts as supplements to a basal G-free diet, and in a third experiment they are tested in combination, using as daily supplements exactly one-half of the amounts of each tested alone. If the vitamin under test consists of two (or more) distinct factors, it would be unlikely that they would be similarly distributed between the two fractions of the vitamin containing material. If they have been partially, if only incompletely, separated by the method of fractionation selected, then the combination of the two fractions may induce a more rapid and a more sustained growth than either of the fractions by itself. If the superiority of the combination of fractions is statistically demonstrable, then a supplementary rather than an additive relation between the fractions has been established, and the conclusion has been justified that the vitamin under study possesses a multiple nature. But if the combination is no better than one of its fractions (in double amount), or particularly if it is midway in value between both, the interpretation is complicated. However, such indecisive results afford no evidence that the vitamin is a single entity.

Sherman and Axtmayer emphasized their use of a 'more quantitative method' of attacking their problem "to the end that the results thus obtained might be more conclusive as to the multiple nature of vitamin B." Their results were in line with those of a number of previous studies in indicating

the complexity of the old vitamin B, and both Stiebeling and Page have submitted evidence of like significance for the multiple character of vitamin G.

This method of Sherman is quantitative to the extent that the intake of vitamin supplements is quantitatively controlled, but it is not quantitative with reference to the intake of the basal diet, containing all other nutrients, since the basal diet is fed ad libitum. The amount of it voluntarily consumed by the experimental rats is neither reported nor in any way considered in the interpretation of the results.

The advantages of controlling the intake of basal diet as well as of the test supplements are real and demonstrable. A variable intake of basal diet with a constant intake of supplement will demonstrably contribute to the variability of the gains in weight secured. Hence, the precision of the average gain secured would be increased if the intake of basal diet as well as of test supplement is equalized. When two qualitatively different supplements are being compared with reference to their growth-promoting power, another source of confusion is introduced if the two supplements possess a differential effect upon appetite, because this differential effect will induce a differential consumption of the basal diet. It is true that the more potent growth-promoting supplement will in general stimulate appetite to the greater extent, but this is not true without exception, and when not true the average gains in weight induced by the two supplements may give a false conception of their relative inherent values in correcting the nutritive deficiency of the basal diet.

If the appetite stimulation of the test supplements parallels their growth-promoting powers, equalization of the intake of the basal diet by the experimental animals, preventing any expression of the appetite effect, will not obscure the growth effect, but will identify it as such beyond question. The separate observation and measurement of the various factors influencing animal growth simplifies the interpretation of the results obtained, if it is not essential, in all cases, to any definite interpretation. If a test supplement exerts an effect

only upon appetite, its dispensability as a participant in animal metabolism can only be proved by complete control of the food intake of experimental animals.

The greater technical precision attainable in vitamin tests by the complete control of the food intake of experimental animals was demonstrated by Garrett and Mitchell ('33). In an application of the paired-feeding technic to vitamin A assay, they were able to show statistically[1] for their particular basal diet, and using eight pairs of rats in testing each ration, that an inclusion of 4.0 per cent or less of a sample of butterfat provided inadequate amounts of vitamin A for maximum growth, while an inclusion of 4.5 per cent or more provided adequate amounts. The method is thus capable of detecting a difference in growth-promoting value of rations containing no more than a 12 per cent difference in vitamin A content. Smaller differences were not tested. With their method of uncontrolled feeding of the basal diet, Sherman and Batchelder ('31), on the basis of questionable 'interpolations' between experimental observations, claim only that "a decrease of 25 per cent (or an increase of 33 per cent) is undoubtedly measurable," while Coward's ('33) results would indicate an even less precise decision.

An improvement in the precision of the Sherman method for detecting the multiple nature of a vitamin would appear to be to make the comparisons of the growth promoting value of the fractionated supplements (2a, 2b, a + b) according to the paired feeding technic, pair mates to receive the same amount of basal diet but different supplements.

This presumably improved technic was applied to the problem attacked by Page ('34) relative to the multiple nature of vitamin G. The procedure of Page was followed in all details, with few exceptions other than the method of feeding. The food source of vitamin G was dried skim milk powder, and the two fractions were prepared, as directed in Page's paper, by extracting the powder with 93 to 94 per cent ethyl

[1] Assuming that a probability of 0.05 or less that the outcome of a comparison is a chance result may be neglected.

alcohol (by weight), concentrating the extract to a small volume, and precipitating with an equal volume of ethyl ether. The two fractions tested for supplementing value were 1) the extracted skim milk powder, and 2) the ether precipitate. The basal diet was the vitamin G-free diet of Bourquin and Sherman. Feeding the basal diet ad libitum to rats that survived a depletion period of 56 days, Page observed (in groups of four animals each) that 1) a daily supplement of 0.3 gm. of the alcohol-extracted milk powder merely maintained the animals at practically constant weight throughout the feeding period of 5 weeks, 2) a daily supplement of 0.07 gm. of the ether precipitate induced an average weekly gain of $6.4 \pm 0.3$ gm., and 3) a daily supplement of 0.15 gm. of the alcohol-extracted milk powder and 0.035 gm. of the ether precipitate induced an average weekly gain of $5.7 \pm 0.4$ gm., not statistically to be distinguished from the gain on 0.07 gm. of the ether precipitate. Page interprets these results as follows:

> In the present stage of our knowledge, the only satisfactory explanation of this supplementary relation between the 2 fractions would seem to be that a long depletion period (56 days) results in depleting the bodily reserves of the rats of a second limiting factor. The first limiting factor is carried by both the ether precipitate and the alcohol extracted milk powder, the second factor by the ether precipitate only. This interpretation is applied only as a suggestive hypothesis which best fits the experimental facts so far available.

However, it would appear that the evidence as thus presented for the existence of two factors in vitamin G is to some extent indecisive, since the results may be explained on the assumption, which cannot be controverted from the data, however improbable it may be, that 0.3 gm. of the extracted milk powder contains a small and inadequate amount of G, but that 0.035 gm. of the ether precipitate contains an adequate amount of G. Hence in order to prove beyond dispute the multiple nature of G, the combination of fractions should be superior in growth promoting value to either fraction when fed separately in double amount.

In repeating Page's study, his directions were followed faithfully with the following exceptions: The paired feeding technic was used in place of ad libitum offering of the basal diet. The same amounts of the same supplements were used, but the caloric value of the supplements was equalized as closely as possible by giving daily as much extracted corn starch as was needed to bring the dry matter of the daily supplements in all cases to 0.3 gm., the size of the largest supplement, i.e., the extracted milk powder. No coprophagy harness was used; however, the rats were kept upon ½ inch mesh wire screening. To maintain the appetite of the rats and to prevent the occasional appearance of neuritic symptoms, it was found necessary to give to each rat 1 drop of tiki-tiki extract twice a week. This necessary addition, indicating that the basal diet did not contain an adequate concentration of vitamin B in spite of its content of an alcoholic extract of wheat, was disturbing, but would not vitiate the results unless the small amounts of tiki-tiki extract consumed contained a G component in adequate amounts for maximum growth. Thus supplemented, the diet permitted the development of growth promoting distinctions between preparations made from milk powder that would contain little if any vitamin B, because of the much greater solubility of B than of G in strong alcohol, and because of the solubility of vitamin B in ethyl ether (Cooper, '12; Block and Cowgill, '32).

The results of the experiment, which was continued for 5 weeks, are summarized in table 1. It is clear from this summary that no evidence was obtained that the alcohol extracted milk powder and the ether precipitate of the alcohol extract supplemented each other as sources of G potency. The ether precipitate was distinctly superior in growth promoting value to the alcohol extracted milk powder, and was probably superior to the combination of one-half portions of each. This combination, in turn, was superior to the alcohol extracted milk powder. The results are fully explained on the supposition that the basal diet is deficient in only one indispensable factor, vitamin G, present in the ether precipitate in

TABLE 1

*Statistics of the paired feeding comparison of the growth promoting value of alcohol extracted skim milk powder (A) and the ether precipitate of the alcohol extract (B) as supplements to a vitamin G-free diet*

| SUPPLEMENTS FED DAILY | | NUMBER OF PAIRS OF RATS | AVERAGE DAILY INTAKE OF BASAL DIET | NUMBER OF FOOD REFUSALS ON | | AVERAGE TOTAL GAIN ON | | DIFFERENCES IN TOTAL GAIN BETWEEN PAIR MATES | | | DIFFERENCES IN BODY LENGTH BETWEEN PAIR MATES | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Number 1 | Number 2 | | | Suppl. 1 | Suppl. 2 | Suppl. 1 | Suppl. 2 | Mean[1] | Standard deviation | Probability[2] | Mean[1] | Standard deviation | Probability[2] |
| | | | *gm.* | | | *gm.* | *gm.* | *gm.* | *gm.* | | *mm.* | *mm.* | |
| 0.3 gm. of A | 0.07 gm. of B | 6 | 3.73 | 164 | 44 | 7.3 | 12.3 | + 5.00 | 2.31 | 0.0024 | + 3.67 | 2.49 | 0.011 |
| 0.3 gm. of A | 0.15 gm. of A<br>0.035 gm. of B | 6 | 3.88 | 90 | 64 | 10.5 | 14.7 | + 4.17 | 2.19 | 0.0041 | + 0.67 | 1.79 | 0.23 |
| 0.07 gm. of B | 0.15 gm. of A<br>0.035 gm. of B | 8 | 3.87 | 48 | 143 | 22.9 | 19.5 | — 3.37 | 4.95 | 0.058 | — 1.50 | 6.34 | 0.22 |

[1] The positive sign indicates a greater gain on supplement no. 2. A negative sign, the reverse.

[2] Probability that fortuitous factors alone determined the differences between pair mates. If the probability is 0.05 or less, according to R. A. Fisher ('28, p. 45), it may reasonably be neglected.

considerably greater concentration than in the alcohol extracted residue. The probabilities given in table 1 (and also those in table 2) were computed from the table of 'Student' ('08).

The experiment possesses a negative significance. It seemed profitable and important to determine whether the method is capable of producing positive results where positive results are to be expected from available evidence. One of the experiments of Sherman and Axtmayer was therefore repeated by the revised method above illustrated. In this experiment a basal ration free of the old vitamin B complex was supplemented, first, with daily portions of 0.8 gm. of ground whole wheat, second, with daily portions of 0.8 gm. of autoclaved yeast, and third, with a combination of 0.4 gm. of each. With uncontrolled feeding of the basal diet, Sherman and Axtmayer were able to demonstrate a superior growth promoting value of the combined supplements to that of either supplement fed in double amount. This outcome constitutes additional evidence that the old B complex is multiple in nature.

This experiment was repeated by the revised fully quantitative technic, except that a comparison of the wheat and autoclaved yeast supplements with one another was omitted as unnecessary. In all other respects the experiment was patterned after that of Sherman and Axtmayer. However, the gains are computed, not on the basis of the final live weights, but on the basis of the final empty weights, after removal of the intestinal contents. These gains in weight are presumably more representative of the actual gains in body tissue. The results obtained are summarized in table 2. It is evident that the combination of wheat and autoclaved yeast was demonstrably superior to either supplement alone in double quantity. This constitutes conclusive evidence, both of the multiple nature of the old B complex, and of the reliability of the feeding method employed in developing positive evidence where the facts warrant.

The greater precision (accuracy) of the paired feeding technic, involving control of the intake of basal ration as well

TABLE 2

*Statistics of the paired feeding comparison of the growth promoting value of ground whole wheat (A) and of autoclaved yeast (B) as supplements to a diet deficient in both vitamins B and G*

| SUPPLEMENTS FED DAILY | | NUMBER OF PAIRS OF RATS | AVERAGE DAILY INTAKE OF BASAL DIET | NUMBER OF FOOD REFUSALS ON | | AVERAGE TOTAL GAIN ON | | DIFFERENCES IN TOTAL GAIN BETWEEN PAIR MATES | | | DIFFERENCES IN BODY LENGTH BETWEEN PAIR MATES | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Number 1 | Number 2 | | | Suppl. 1 | Suppl. 2 | Suppl. 1 | Suppl. 2 | Mean[1] | Standard deviation | Probability[2] | Mean[1] | Standard deviation | Probability[2] |
| | | | gm. | | | gm. | gm. | gm. | gm. | | mm. | mm. | |
| 0.8 gm. of A | 0.4 gm. of A<br>0.4 gm. of B | 8 | 3.14 | 183 | 16 | 6.2 | 10.4 | + 4.25 | 1.39 | < 0.0001 | + 4.37 | 2.29 | 0.0008 |
| 0.8 gm. of B | 0.4 gm. of A<br>0.4 gm. of B | 8 | 3.14 | 120 | 68 | 17.1 | 19.0 | + 1.87 | 2.31 | 0.035 | + 1.75 | 2.68 | 0.066 |

[1] The positive sign indicates a greater gain on supplement no. 2. A negative sign, the reverse.

[2] Probability that fortuitous factors alone determined the differences between pair mates. If the probability is 0.05 or less, according to R. A. Fisher ('28, p. 45), it may reasonably be neglected.

as of vitamin supplement, over that of the method of Sherman and Axtmayer, involving control of the intake of vitamin supplements only, may be assessed from the data in these five paired feeding comparisons. This may be done by computing in two ways the probability that chance alone would produce a group difference as great as or greater than that observed in each of the comparisons of two vitamin supplements. By the first method, the probability is based upon the standard deviation of the eight differences in gain between paired rats, the method followed in a 'Student' analysis. These probabilities are given in tables 1 and 2. By the second method, the probability is computed from the standard deviation of the total gains exhibited by the rats on each of the two supplements according to a method proposed by Fisher ('28, p. 107). In this method no advantage is taken of the equalization of intake of basal diet by paired rats. If this modification in vitamin assay work is effective in increasing the precision of the test, the probabilities (P) computed by the first method should be much less than the probabilities computed by the second method. In such case the proposed modification would be a refinement in technic. But if the complete control of food intake is ineffective in accomplishing its purpose, then the probabilities computed by the second method should be less than those computed by the first method. As Fisher says:

> In cases in which each observation of one series corresponds in some respects to a particular observation of the second series, it is always legitimate to take the differences and test them . . . . as a single sample; but it is not always desirable to do so. A more precise comparison is obtainable only if the corresponding values of the two series are positively correlated, and only if they are correlated to a sufficient extent to counterbalance the loss of precision due to basing our estimate of variance upon fewer degrees of freedom.

The values of P[2] computed by the two methods for the five comparisons given in tables 1 and 2 are, in order, 0.0024 and 0.081, 0.0041 and 0.068, 0.058 and 0.11, less than 0.0001 and 0.019, 0.035 and 0.35. It is clearly evident that the precision

[2] In using Fisher's table of t values, the corresponding values of P should be halved to correspond to the 'Student' probabilities (see p. 105 of Fisher's book).

of the assay has been greatly improved by the equalization of the intake of basal diet between comparable animals.

Perhaps some quantitative idea of the increase in precision of the paired over the unpaired comparison of gains may be obtained from the ratio of the standard deviations of the mean difference in gain between each pair of supplements as computed, first, from the standard deviations of the gains made on each supplement, and, second, from the paired differences in gain. This ratio varied from 1.01 to 3.8 and averaged 2.3. Hence, it may be concluded that the equalization of the intake of basal diet for comparable animals increased the precision of the comparison of the growth promoting value of vitamin supplements 2.3 times.

The complete equalization of the food intake of comparable animals thus greatly improves the accuracy of vitamin assay methods involving growth measurements only. It is not unreasonable to expect that it would improve also those vitamin assay methods concerned with the prevention or the cure of deficiency disease symptoms. Clear indications to this effect are the findings of Amantea ('33) and of Westenbrink ('34) that the time of appearance of polyneuritis in pigeons is directly dependent upon the amount of vitamin B-free diet consumed, and those of Watkins and Mitchell ('35) that the more rachitogenic diet rats consume the quicker do rachitic symptoms develop.

## CONCLUSIONS

Both the precision and the clarity of vitamin assay methods are improved by controlling the consumption by the experimental animals of the basal diet, as well as of the vitamin supplements. In particular, the Sherman and Axtmayer method of determining whether or not a given vitamin possesses a multiple nature, is rendered completely quantitative by extending the control of food intake to the basal diet. The precision of the comparison of the growth promoting value of two vitamin supplements is increased more than twice by this modification, and any ambiguity in interpretation occasioned by a differential effect upon appetite of different vita-

min fractions, whether containing single or multiple supplementing factors, is removed.

Applying this modified Sherman and Axtmayer method to two G fractions prepared from skim milk powder (according to the directions of Page), no evidence of the multiple nature of the vitamin was obtained.

## LITERATURE CITED


AMANTEA, G. 1933 Sul fattore antineuritico ($B_1$) e sul concetto di 'quoziente beri-beri (Qb).' Rend. R. Accad. Naz. Lincei, Cl. Sci., vol. 18, pp. 317–323 (cited in Nutr. Abstracts and Revs., vol. 3, p. 986, 1934).

BAUMANN, C. A., B. M RIISING AND H. STEENBOCK 1934 Fat soluble vitamins. XL. The growth method of determining vitamin A. Zeitschr. f. Vitaminforschung, vol. 3, pp. 81–88.

BLOCK, R. J., AND G. R. COWGILL 1932 The antineuritic vitamin. IV. The preparation of a highly potent concentrate. J. Biol. Chem., vol. 98, pp. 637–643.

COOPER, E. A. 1912 The protective and curative properties of certain foodstuffs against polyneuritis induced in birds by a diet of polished rice. J. Hygiene, vol. 12, pp. 436–462.

COWARD, K. H. 1933 The influence of the length of the test period on the accuracy obtainable in a vitamin A test. Biochem. J., vol, 27, pp. 445–450.

FISHER, R. A. 1928 Statistical Methods for Research Workers. 2nd ed. Edinburgh and London. 269 pp.

GARRETT, O. F., AND H. H. MITCHELL 1933 An application of the paired feeding method to the quantitative estimation of the relative vitamin A contents of foods and artificial concentrates. Am. J. Physiol., vol. 104, pp. 608–614.

PAGE, J. W., JR. 1934 Experiments on vitamin G concentration and possible supplementary relationships with the vitamin G deficient diet. Proc. Soc. Exper. Biol. and Med., vol. 31, pp. 779–783.

SHERMAN, H. C., AND J. H. AXTMAYER 1927 A quantitative study of the problem of the multiple nature of vitamin B. J. Biol. Chem., vol. 75, pp. 207–212.

SHERMAN, H. C., AND E. L. BATCHELDER 1931 Further investigation of quantitative measurement of vitamin A values. J. Biol. Chem., vol. 91, pp. 501–511.

STIEBLING, H. K. 1931–1932 Further evidence of additional substances essential to mammalian nutrition. Proc. Soc. Exper. Biol. and Med., vol. 29, pp. 1155–1159.

'STUDENT' 1908 The probable error of a mean. Biometrika, vol. 6, pp. 1–25.

WATKINS, W. E., AND H. H. MITCHELL 1935 The phosphorus requirements of growing chickens, with a demonstration of the value of controlled experimental feeding. To appear soon in Poultry Science.

WESTENBRINK, H. G. K. 1934 Über den Einfluss der Menge und der Zusammenstellung der Nahrung auf die Folgen von Vitamin $B_1$-Mangel. Arch. néerland. physiol., vol. 19, pp. 94–115; Ber. ges. Biol., Abt. B: Ber. ges. Physiol. u. exp. Pharmakol., vol. 79, pp. 585–586.

# THE INFLUENCE OF VITAMIN C LEVEL UPON RESISTANCE TO DIPHTHERIA TOXIN[1]

## I. CHANGES IN BODY WEIGHT AND DURATION OF LIFE

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TWO FIGURES

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It is difficult to evaluate the physiological significance of the milder types of malnutrition, but it is reasonably certain that this phase of nutrition studies is closely related to human dietary problems. The investigations of Sherman ('32) and McCarrison ('21) have been particularly fruitful in showing the influence of deficiencies in the zone where there is no obvious manifestation of a 'deficiency disease.' A good review of the relation between malnutrition and resistance against infections has been published recently by Clausen ('34).

In the course of making repeated assays of vitamin C concentrates over a period of several years previous to its isolation and identification, it was consistently noted that there was a fairly wide zone of latent deficiency in which there was a marked loss of body tone without exterior indications of scurvy. During assays with the pure vitamin (Waugh and King, '32) from different natural sources and with the synthetic vitamin,[2] the same characteristic zone was

[1] Contribution no. 296 from the department of chemistry and from the pathology department. A summary of this paper was presented before the American Institute of Nutrition, April 10, 1935 at Detroit, Michigan.

[2] The synthesis was carried out through sorbitol sorbose sorbosone 2-keto-l-gulonic acid vitamin C, by Dr. E. G. White in these laboratories during 1933–1934.

observed. The finding of a high concentration of the vitamin in glandular tissue (Bessey and King, '33) together with its known intimate relation to growth, tooth and bone development, capillary strength, collagen gelation (Wolbach and Howe, '26; Menkin, Wolbach and Menkin, '33), resistance to infection (Sherman and Smith, '31), cellular respiration (Szent-Gyorgyi, '30, '31; Quastel and Wheatley, '34) and other enzyme systems (Karrer and Zehender, '33; Purr, '33, '35) gave further evidence of its probable importance in physiological processes not associated specifically with scurvy. In other words, physiological impairment from a moderate vitamin deficiency might reasonably become evident in a manner not directly associated with the usual symptoms of a complete or more drastic vitamin deficiency.

In the present investigation we have studied the resistance of guinea pigs to standardized diphtheria toxin when the animals were on different levels of vitamin C intake. The main purpose of the study was to find whether or not there is a significant lowering of resistance to the toxin in the zone of vitamin C deficiency where the typical symptoms of scurvy are not well developed (generally referred to as 'latent scurvy').

Diphtheria toxin was selected because: a) it was available as a standardized product, and b) the tissue changes induced by diphtheria toxin injections are similar in certain respects to those observed in scorbutic guinea pigs (Bessey, Menten and King, '34). It has the additional advantage of being a toxin frequently encountered in human pathology.

Rinehart and Mettier ('34) claim that there is an intimate relation between 'latent scurvy' in guinea pigs and the effect of streptococcic infections. Discordant results were found clinically, however, at the Rockefeller Institute (Schultz, Sendray and Swift, '35). During the course of the present investigation, Harde and associates ('34, '35) have reported interesting relationships between vitamin C and diphtheria toxin, with additional notations on other types of toxins arising from infections.

## EXPERIMENTAL

*Procedures.* Male guinea pigs (purchased) about 250 gm. in weight, were placed in individual cages and given the usual care observed when making vitamin C assays. During a preliminary period they were given a standard vitamin C-free (Sherman) diet (bran, oats, milk powder, butter fat, and salt, supplemented by yeast and cod liver oil) and fresh spinach, to permit them to reach an average weight of about 300 gm. and to assure a normal growth rate and apparent good health at the beginning of the experiment. They were then divided into groups whose average weights and general condition were as nearly comparable as possible. Each animal was then given a definite quantity of vitamin C daily from a graduated pipette, using standardized orange juice (2,6-dichlorophenol-indophenol titration) or a standard solution of the pure vitamin. The animals were examined practically daily and were autopsied at death or at the end of each series of tests.

Dilutions of diphtheria toxin[3] in sterile saline were injected subcutaneously near the groin. Because of severe edema, sloughing and occasional local infections in animals on low vitamin C levels, it was found preferable to make the injections on either side of the spinal column.

Tissue respiration measurements were made on liver and kidney tissue[4] from representative animals. In each instance the animals were killed by a blow over the occiput. The tissue was immediately suspended in Locke's solution without dextrose. Determinations were made on 100 mg. finely cut portions in duplicate or triplicate with a Barcroft-Warburg constant volume manometric apparatus. The oxygen consumption from air was recorded at 30-minute intervals over 2-hour periods.

Titrations of vitamin C were carried out in the presence of 8 per cent trichloroacetic acid, using 2,6-dichlorophenol-indophenol as described in an earlier paper (Bessey and

[3] The toxin was obtained from Parke, Davis & Co.

[4] The respiration measurements were made by Mr. Martin Yavorsky with the apparatus at the U. S. Bureau of Mines through the courtesy of Mr. W. P. Yant.

King, '33). A carefully washed suspension of $Al(OH)_3$ was found helpful for decolorizing and clarifying extracts from liver, spleen, and kidney tissue.

## RESULTS AND DISCUSSION

In preliminary experiments[5] it was found that single injections of diphtheria toxin containing 1.5 or 2.0 M.L.D. produced such extensive lesions that there was no satisfactory basis for differentiating between the effects of low and high vitamin intake. With single injections of 1.0 M.L.D., however, the survival time of five animals receiving 5 mg. of pure vitamin daily was greater (average 50 per cent) than that of four animals depleted until mild indications of scurvy were evident. In later experiments, with smaller repeated doses of toxin (0.1, 0.3 and 0.5 M.L.D.), the differences between latent scorbutic and normal animals became clearly evident, as shown in the detailed records which follow.

Degeneration with loss of lipoid constituents and frequently hemorrhage of the adrenal cortices was evident as a result of either vitamin C depletion or injection of diphtheria toxin.

Titration of the vitamin C in liver, spleen, kidney and adrenal tissue of six animals receiving toxin compared to five animals on the same vitamin level without toxin did not indicate significant changes in vitamin content due to toxin injections. The titrations were made approximately 3 weeks subsequent to the injections, however, and the intermediate period might have permitted readjustments subsequent to earlier changes.

Table 1 provides a summary of the findings with thirty-eight animals in relation to survival and changes in body weight in a single experiment. Groups A and B, with fifteen animals each, received 0.50 and 0.25 mg. of vitamin C per day (given orally as orange juice), representing respectively, minimum protective and sub-protective levels. Two groups, $A^1$ and $B^1$, with four animals each on the 0.50 and 0.25 mg.

[5] The authors wish to acknowledge the assistance of Dr. O. A. Bessey during the preliminary part of the work in 1933, and of Messrs. J. H. Leathem, M. Kochin and S. C. Camp in the later experiments.

levels served as controls, without toxin. For 10 days previous to the test period, all animals received 1.5 mg. per day. Animals in groups A and B were given subcutaneously 0.3 M.L.D. of toxin on the eighth, eleventh, twenty-fifth and twenty-ninth days after being put on their respective vitamin levels. Their minimum, average and maximum weights at the time of the first injection were 260, 325 and 418 gm. for group A, and for group B the corresponding values were 257, 323 and 406 gm. It will be noted that within 26 days after the first injection, the number of deaths was twice as great on the lower nutritive level (0.25 mg. per day) as on the higher level

TABLE 1

*Resistance of guinea pigs to diphtheria toxin when on different levels of vitamin C intake*

| GROUP AND NUMBER OF ANIMALS | MILLIGRAMS OF VITAMIN PER DAY | TOTAL TOXIN FOUR INJECTIONS | NUMBER OF DEATHS WITHIN 26 DAYS | NUMBER SURVIVING AFTER 55 DAYS | AVERAGE SURVIVAL AFTER SECOND INJECTION | AVERAGE CHANGE IN WEIGHT GRAMS 55 DAYS |
|---|---|---|---|---|---|---|
| | | | | | *days* | |
| (A) 15 | 0.50 | 1.2 M.L.D. | 5 | 3 | 32[1] | −54 |
| (B) 15 | 0.25 | 1.2 M.L.D. | 10 | 0 | 21 | −115[2] |
| (A[1]) 4 | 0.50 | 0 | 0 | 4 | .. | +187 |
| (B[1]) 4 | 0.25 | 0 | 0 | 4 | .. | −30 |

[1] Exclusive of three animals which survived the entire period and were rapidly gaining in weight at the end of the test.

[2] Average net change in weight at time of death.

(0.50 mg. per day). Three animals on the 0.5 mg. level survived the entire experimental period and were gaining in weight at the end of the test. The contrast in severity and duration of subcutaneous reactions at the point of injection was striking. Animals on the higher level showed marked swelling and local congestion for several days, following which there was slow healing action. Those receiving 0.25 mg. developed more severe swelling, necrosis and hemorrhage at the site of injection. These differences were evident before there was any general indication of typical 'scurvy' among those receiving 0.25 mg. per day. At autopsy, every animal on the 0.25 mg. level showed hemorrhagic necrosis at

the site of the first two injections, while only four animals on the 0.50 mg. level showed appreciable congestion at the site of the first injection, and in these four the condition was much less severe than in the 0.25 mg. group. In view of the variations in vitamin C titration values found in tissues of individuals dying from various conditions (Yavorsky, Almaden and King, '34), these results are of interest in relation to human pathology.

A later experiment is summarized in figure 1. Two levels of vitamin intake were again compared. Thirty guinea pigs were used, with five animals in each experimental group.

After 25 days on a basal diet supplemented with spinach, the animals were divided into the six groups and given orange juice as a source of vitamin C. Those represented by curves A, B and C were then given 1.5 mg. of vitamin daily. After being depleted 5 days, those included in curves $A^1$, $B^1$ and $C^1$ received 0.3 mg. of vitamin per day. A and $A^1$ served as control groups for the two vitamin levels, without receiving toxin injections. B and $B^1$ received 0.3 M.L.D. injections of toxin on the sixteenth, twenty-first, twenty-eighth and thirtieth days after being placed on the standard vitamin levels. C and $C^1$ received 0.5 M.L.D. injections of toxin on the same days, i.e., parallel with B and $B^1$. The minimum, average and maximum weights of animals for the different groups at the time of the first toxin injection were as follows: A, 313, 367, 450; B, 333, 348, 360; C, 370, 395, 415; $A^1$, 270, 302, 327; $B^1$, 328, 361, 388; $C^1$, 373, 413, 453.

It will be noted that the group receiving 1.5 mg. of vitamin C daily and 0.3 M.L.D. injections (curve B) suffered only a slight loss in weight, following which they resumed normal growth. When growth was resumed, the local lesions had disappeared except for a small amount of scar tissue. In contrast, those receiving only 0.3 mg. of vitamin per day, and the same amount of toxin (curve $B^1$), were all dead at the end of the experiment and at autopsy showed severe subcutaneous hemorrhagic necrosis.

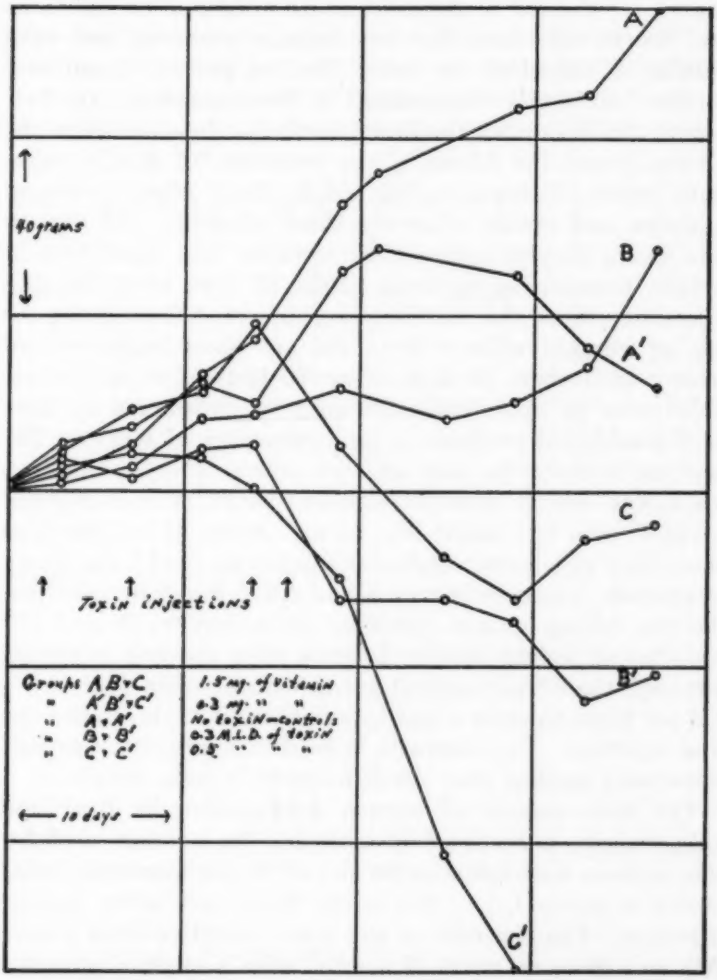


Fig. 1 Effect of vitamin C feeding level upon the resistance of guinea pigs against diphtheria toxin.

The 1.5-mg. group which received 0.5 M.L.D. injections (curve C) showed a marked loss in weight after the third and fourth injections, but two animals survived and were gaining in weight at the end of the test period. Local congestion had nearly disappeared in those surviving the test period (38 days after the first injection). In contrast to the 1.5-mg. group, the 0.3-mg. group receiving 0.5 M.L.D. injections (curve $C^1$) began to lose weight slowly after the second injection and rapidly after the third injection. All pigs in this group showed severe local reactions and rapid loss of weight, terminating in death within 27 days after the first injection. Since the control group (curve $A^1$) receiving 0.3 mg. of vitamin without toxin did not show indications of scurvy until about 10 days after the last injection, distinct differences in toxin resistance may be considered to have been manifested previous to the appearance of scurvy. The contrast between the high and low nutritive levels receiving toxin was clearly evident (compare curve B with $B^1$, and curve C with $C^1$) before the 0.3-mg. group of controls had shown any significant weight deviation from the 1.5-mg. group of controls (compare curves A and $A^1$). It will be seen that the two 0.3-mg. groups receiving toxin (curves $B^1$ and $C^1$) were losing weight rapidly 13 days after the first injection, although the 0.3-mg. control group without toxin (curve $A^1$) did not begin to show a loss in weight until 19 days after the first injection. The contrasts in local inflammatory reactions were more marked than the differences in body weight.

The measurement of oxygen consumption by liver and kidney tissue is recorded in table 2. The animals used for this purpose were taken at the end of the experimental period shown in figure 1, i.e., during the third week after the last injection. The controls on the lower nutritive level (curve $A^1$) and those on curve B showed only a slight depression below the values for the normal group (curve A). The animals from curves $B^1$ and C exhibited a reduction of approximately 15 per cent in oxygen uptake for kidney tissue. Apparatus was available for only two to four animals from each

group. The animals from group $C^1$ died before manometric measurements were started.

To check the results of the previous experiments and to eliminate the possible interference of factors other than vitamin C in orange juice, a further series (summarized in fig. 2) was run, in which the pure vitamin was fed in aqueous

TABLE 2

*Oxygen consumption in cubic millimeters per 100 mg. of wet tissue at end of 2 hours at 37°*

| NUMBER OF ANIMALS | VITAMIN LEVEL | AMOUNT OF TOXIN INJECTED | CORRESPONDING CURVE IN FIGURE 1 | LIVER | KIDNEY |
|---|---|---|---|---|---|
| | mg. | | | | |
| 2 | 1.5 | 0 | A | 90.3 | 201.9 |
| 4 | 1.5 | 4 doses each of 0.3 M.L.D. | B | 91.7 | 193.6 |
| 2 | 1.5 | 4 doses each of 0.5 M.L.D. | C | 75.2 | 175.5 |
| 3 | 0.3 | 0 | $A^1$ | 87.5 | 189.7 |
| 3 | 0.3 | 4 doses each of 0.3 M.L.D. | $B^1$ | 88.0 | 170.4 |

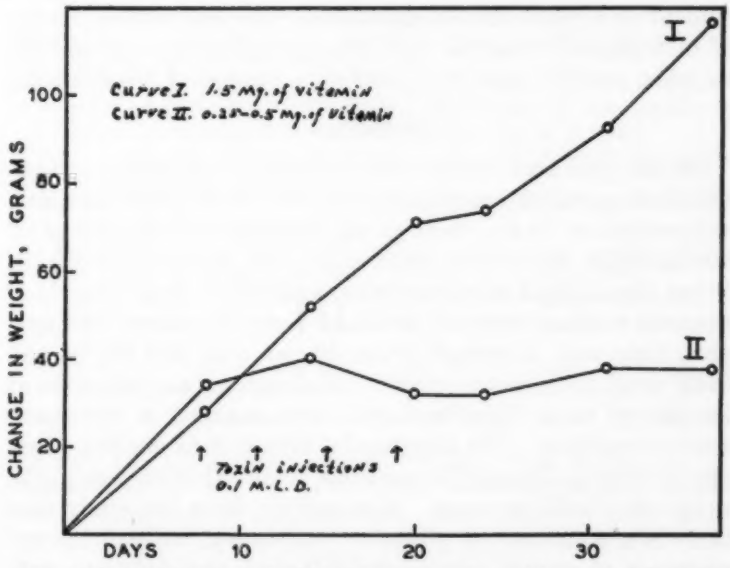


Fig. 2 Effect of vitamin C feeding level upon the resistance of guinea pigs against small dosages of diphtheria toxin.

solution. One group of eight animals (curve I) received 1.5 mg. of vitamin daily. The other group of nine animals (curve II) was depleted for 8 days and then given 0.25 mg. of vitamin C per day for 2 weeks, after which the vitamin intake was increased to 0.50 mg. per day to avoid the onset of scurvy. Each animal of both groups was injected subcutaneously with 0.1 M.L.D. of diphtheria toxin on the fourteenth, seventeenth, twenty-first and twenty-fifth days. The minimum, average and maximum weights of animals represented by curve I at the time of the first injection were 350, 399 and 430 gm. respectively, while the corresponding values for curve II were 325, 377 and 415 gm. Those in curve I (final average weight 464 gm.) showed very little deviation from a normal growth rate, and at the end of the test period showed practically complete healing at the sites of injection. Those in curve II (final average weight 375 gm.) were unable to resume growth when placed upon the 0.5-mg. level, and local congestion was evident as a result of the injections. The contrast in degree of inflammatory reaction and rate of healing was as marked as when orange juice was used as a source of the vitamin.

## SUMMARY

Guinea pigs under controlled conditions and receiving definite daily quantities of vitamin C at abundant, protective, and sub-protective levels, were given subcutaneous injections of standardized diphtheria toxin in 0.1, 0.3, 0.5 and 1.0 M.L.D. When the animals were partially depleted of their vitamin C reserves without showing external signs of scurvy, the survival time was shortened about 50 per cent and the loss in body weight was more severe. Hemorrhage and necrosis at the site of toxin injections were more marked in the latent scurvy condition. The decrease in oxygen consumption capacity of liver and kidney tissue after toxin injection was in the range of 5 to 15 per cent. It is evident from the study that there is a wide zone of vitamin C deficiency, without the appearance of scurvy, where physiological processes are subnormal and the animal is more sensitive to injury from bacterial toxin.

## LITERATURE CITED


BESSEY, O. A., AND C. G. KING 1933 The distribution of vitamin C in plant and animal tissues, and its determination. J. Biol. Chem., vol. 103, p. 687.

BESSEY, O. A., M. L. MENTEN AND C. G. KING 1934 Pathologic changes in the organs of scorbutic guinea pigs. Proc. Soc. Exp. Biol. Med., vol. 31, p. 455.

CLAUSEN, S. W. 1934 The influence of nutrition upon resistance to infection. Physiol. Rev., vol. 14, p. 309.

GREENWALD, C. K., AND E. HARDE 1935 Vitamin C and diphtheria toxin. Proc. Soc. Exp. Biol. Med., vol. 32, p. 1157.

HARDE, E. 1934 Ascorbic acid and intoxications. C. R. de l'Acad. des Sc., vol. 199, p. 618.

HARDE, E., AND M. PHILLIPE 1934 Observations on the antigenic strength of a mixture of diphtheria toxin and vitamin C. C. R. de l'Acad. des Sc., vol. 199, p. 738.

HARDE, E., T. A. ROTHSTEIN AND H. D. RATISH 1935 Urinary excretion of vitamin C in pneumonia. Proc. Soc. Exp. Biol. Med., vol. 32, p. 1088.

HARDE, E., AND H. R. BENJAMIN 1935 Vitamin C content of tissues of laboratory animals under various pathological conditions. Proc. Soc. Exp. Biol. Med., vol. 32, p. 651.

KARRER, P., AND F. ZEHENDER 1933 Vitamin C as activator of catheptic enzymes. Helv. Chim. Acta, vol. 61, p. 701.

MCCARRISON, R. 1921 Studies in Deficiency Diseases, p. 209. Henry Froude and Hodder and Stoughton, London.

MENKIN, V., S. B. WOLBACH AND M. F. MENKIN 1933 Formation of intercellular substance by the administration of ascorbic acid (vitamin C) in experimental scorbutus. Am. J. Path., vol. 10, p. 569.

PURR, A. 1933 The influence of vitamin C on intracellular enzyme action. Biochem. J., vol. 27, p. 1703.

———— 1935 The activation phenomena of papain and cathepsin. Biochem. J., vol. 29, p. 13.

QUASTEL, J. H., AND A. H. M WHEATLEY 1934 An effect of ascorbic acid on fatty acid oxidations in liver. Biochem. J., vol. 28, p. 1014.

RINEHART, J. F., AND S. R. METTIER 1934 The heart valves and muscle in experimental scurvy with superimposed infection. Am. J. Path., vol. 10, p. 61.

SCHULTZ, M. P., J. SENDROY AND H. F. SWIFT 1935 Vitamin C and rheumatic fever. Science, vol. 81, p. 8.

SHERMAN, H. C. 1932 Chemistry of Food and Nutrition, pp. 532–538. The Macmillan Co., New York.

SHERMAN, H. C., AND S. SMITH 1931 The Vitamins. Chem. Cat. Co.

SZENT-GYORGYI, A. 1930 On the mechanism of biological oxidation and the function of the suprarenal gland. Science, vol. 72, p. 125.

———— 1931 On the function of hexuronic acid in the respiration of the cabbage leaf. J. Biol. Chem., vol. 90, p. 385.

WAUGH, W. A., AND C. G. KING 1932 The isolation and identification of vitamin C. J. Biol. Chem., vol. 97, p. 325.

———— 1932 The chemical nature of vitamin C. Science, vol. 76, p. 630.

WOLBACH, S. B., AND P. R. HOWE 1926 Intercellular substances in experimental scorbutus. Arch. Path., vol. 1, p. 1.

YAVORSKY, M., P. ALMADEN AND C. G. KING 1934 The vitamin C content of human tissues. J. Biol. Chem., vol. 106, p. 525.

# THE INFLUENCE OF VITAMIN C LEVEL UPON RESISTANCE TO DIPHTHERIA TOXIN

## II. PRODUCTION OF DIFFUSE HYPERPLASTIC ARTERIOSCLEROSIS AND DEGENERATION IN VARIOUS ORGANS

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ONE PLATE (FOUR FIGURES)

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Repeated attempts to produce in experimental animals a diffuse hyperplastic arteriosclerosis similar to that encountered in human pathology has thus far not met with signal success. In the course of microscopic examination of tissue removed from guinea pigs maintained on diets deficient in vitamin C and treated with diphtheria toxin, as described in the previous paper, hyperplastic vascular lesions were constantly obtained. The microscopic description of these lesions forms the basis of the present communication.

Lack or absence of vitamin C gives rise to definite degenerative changes in the highly specialized organs as well as developmental irregularities in bones and teeth. Among the former may be cited granular degeneration with hemorrhage in the adrenal cortex, fatty degeneration and hemorrhage in the cardiac musculature, central toxic necrosis of liver, atrophy of germinal epithelium of the testis and ovary, ulcers of the intestinal mucosa, and degeneration, hemorrhage and atrophy throughout the entire skeletal musculature. These have been described by several investigators (Bessey, Menten and King, '34; Meyer and McCormick, '28; Lindsay and Medes, '26). The addition of bacterial toxins to the circulation hastens and intensifies the development of such processes

but it is not our purpose to give a detailed amplification of such degeneration. It is desired rather to describe an additional phenomenon, which results when the guinea pig, an animal incapable of synthesizing vitamin C, is maintained on an inadequate vitamin C diet, and coincidently subjected to the continuous action of diphtheria toxin. A measure of the general effect of such a regime has already been indicated in the loss of weight and decreased duration of life reported in the previous paper.

## TECHNIC AND RESULTS

The material obtained was removed at necropsy from the various animals, whose treatment has been outlined in the previous paper. Tissues fixed immediately after death in Zenker's solution and 10 per cent formalin were routinely stained with hematoxylin and eosin. This stain was supplemented by Sudan iv and Verhoeff's and Weigert's elastic tissue stain. Although more or less degenerative change occurred throughout the body, only lesions in lung, heart, liver, spleen, kidney, pancreas and brain will be described. Hyperplastic arteriosclerosis was observed in all of these except the heart and brain.

The lungs were fairly normal except for degeneration in the bronchial wall, many thickened vessels and frequently irregularly distributed areas of collapsed lung tissue, whose blood supply had been materially reduced by arteriosclerotic vessels. Sclerotic vessels were the outstanding pathologic feature of microscopic sections of lung. Indeed, this vascular lesion was better demonstrated in lung than in any other organ. The enormously thickened vessels, the walls of which had impinged upon and decreased the lumina to a minimum, contrasted strikingly with the surrounding loose alveolar pulmonary tissue (figs. 1 and 2). The thickening was most marked in the medium-sized arterioles but was also observed in larger arteries. The thickening was due to a diffuse hyperplasia of the connective tissue, mainly of the media. It was fairly uniform and continuous except at the point of origin of

the smaller branches. Occasionally the hyperplasia did not include the entire circumference but was lacking on that side of the vessel which was buttressed by the contiguous bronchiole. The internal elastic lamina was swollen, but retained a considerable affinity for the elastic tissue reagents. In some of the smaller arteries the widespread degeneration of the elastic tissue had rendered the identification of the internal elastic lamina impossible and when such vessels underwent hyperplasia it was difficult to apportion the amount belonging to media or intima. In many such vessels the degeneration throughout had been so rapid and extensive that compensatory hyperplasia had not taken place. Although the retrograde change in the elastic tissue was the conspicuous feature, the earliest change was in the smooth muscle. Of all the medial elements the muscle cells appeared to be the most susceptible to the toxin and it was in them that the pathologic process was initiated.

The first detectable microscopic change was a cloudy swelling gradually progressing to a necrosis with pyknosis and loss of staining of the smooth muscle cell. With large doses of toxin the disintegration of the muscle cells was often so rapid that no trace of the process was discernible. Sometimes a few remnants of pyknotic nuclei remained. The next step in the retrograde process was the involvement of the elastic tissue. These fibers began to take on a granular appearance with loss of their distinct marginal outline. Variations in the degree of staining with Verhoeff's and Weigert's elastic tissue stains began to appear. The fibers lost their wavy contour and tended to lie in straight rows. Some of the fibers did not stain at all. Finally, clumping and disintegration took place and many of the elastic fibers disappeared completely. The process was one of gradual deterioration. Splitting and fraying of the elastic tissue were not observed. These changes were not accompanied by an appreciable fat phanerosis. Furthermore, calcification did not occur.

The early disappearance of smooth muscle associated with loss of resiliency and tone in the elastic fibers induced a compensatory hyperplasia of the connective tissue of the arteries.

In the larger vessels the retrograde changes began in the central zone of the media, and then advanced inward and outward. When the entire media became involved there was often a thinning of the vessel wall, due to compression of the residual elastic tissue fibers so that they were pressed and clumped together. Often between these could be seen necrobiotic granular tissue which stained irregularly. This was constantly seen in the wall of the aorta and pulmonary vein. Compensatory fibrosis does not occur in these vessels, but an attempt to counteract this loss of supporting tissue and reduction in resiliency was seen in the formation of small nodules of cartilage. These were accessory to the aortic nodules found in older pigs and were usually situated at a greater distance from the roots of these vessels. Metaplasia of connective tissue into cartilage could be seen in various stages. Although the vascular lesions are of the productive instead of the degenerative type produced by Klotz ('06), Bailey ('17), and Duff ('32) with diphtheria toxin in rabbits, they possess certain features described by these authors. The degenerations taking place in smooth muscle and elastic tissue are common to both. The intimal changes described by them in the aorta were not seen. The small size of the guinea pig's aorta may have made these inconspicuous, and hence overlooked. The same etiologic factors preceding arterial thickening, namely, loss of smooth muscle and degeneration of the elastic fibers are responsible for the thinning of the bronchial wall with subsequent crumpling, buckling and infolding, occasionally seen in sections. Compensatory fibrosis was lacking and the degenerations in these structures were often accompanied by retrograde changes in the contiguous lymphocytic nodules which stain very intensely.

Myocardial degeneration resulted in widespread loss of heart tissue which manifested itself in the grossly shrunken and wrinkled visceral pericardium. The progressive stages beginning with loss of cross striation and ending with complete dissolution of the muscle cell could be followed in various parts of the heart. Many small collapsed arterioles remained as closely approximated lines of pyknotic nuclei.

Their closure added to the rapid dissolution of areas of myocardial substance. The visceral pericardium occasionally showed slight degrees of fibrosis or fatty infiltration. The valves did not show significant changes. In some cases there was a slight medial thickening of the larger coronary vessels near the base of the heart which was probably unrelated to the experimental procedure. This was the only evidence of hyperplastic arteriosclerotic change in the myocardium observed in any of the animals. This lack of sclerosis and the presence of collapsed degenerated capillaries in the heart, is in sharp contrast to the marked vascular thickening occurring in the lungs.

In the liver the arterial thickening introduced an additive factor in the progressive cellular degeneration and lobular necrosis obtaining in scorbutic and sub-scorbutic conditions. The hyperplasia of the radicles of the hepatic artery never attained the extent seen in the pulmonary vessels. This can probably be partly explained by the resistance offered by the surrounding dense liver tissue. Also, only comparatively few of the interlobular vessels were thickened. The walls of the thickened interlobular arteries were fairly uniform in thickness and had a hyaline appearance. Their lumina were frequently so reduced that they were almost occluded (figs. 3 and 4).

In the spleen the arteriosclerosis was best seen in those branches of the splenic artery encircled by the lymphatic sheaths, especially in the center of the malpighian corpuscles. The central vessels of many of these structures, however, showed no hyperplasia and the distribution of arteriosclerosis was irregular. The appearance of the thickened vessels was similar in size and appearance to those seen in the liver.

The distribution of the sclerotic vessels in the kidney was irregular. It was difficult to determine with certainty in which of the smaller divisions of the renal artery the hyperplasia had taken place. The interlobular arteries and arteriolae rectae were apparently constantly involved. In a few instances the widespread cellular degeneration result-

ing in reduction of the number of secreting units, with compensatory dilatation of the remaining tubules suggested that the entire arterial tree had been involved. The destructive quality of the toxin per se in producing similar retrograde process in renal parenchymatous tissue should be kept in mind in evaluating the consequences of vascular occlusion. In only one animal were morphologic changes in the glomeruli observed which approximated those seen in the human arteriosclerotic kidney. The occluded glomeruli in this case, however, appeared hyaline resembling some of the sclerotic hepatic vessels.

In those pigs receiving small repeated injections of diphtheria toxin productive vascular lesions were not found. Microscopic examination was made on tissue from six pigs maintained on 0.25 mg. vitamin C daily and given 0.1 M.L.D. diphtheria toxin on the fourteenth, seventeenth, twenty-first and twenty-fifth day after the beginning of the deficient diet (see fig. 2 previous paper). Two of the pigs, killed 1 day after the fourth injection, showed a moderate thickening in some of the larger pulmonary arteries. The vessels of the remaining four pigs, killed 16 days after the last injection, appeared normal. From these observations it is inferred that such dosage is not sufficient to produce vascular sclerosis. It is possible that small repeated injections made at 3- or 4-day intervals may induce a degree of immunity against the diphtheria toxin which modifies its toxic effect.

Evidence of arteriosclerosis in the pancreas was observed in a few medium-sized vessels. The picture here resembled that described in pulmonary vessels of similar size. The readiness with which vascular thickening can be produced with diphtheria toxin in guinea pigs and especially in vitamin C-deficient pigs would suggest that some evidence of hyperplastic arteriosclerosis might be found in normal pigs which are fairly susceptible to spontaneous infections. Medial fibrosis of a slight degree was observed in the large pulmonary vessels of some of the control animals. This was more conspicuous in two or three of the pigs which had been

maintained on a scorbutic diet and we believe can be attributed to a concurrent infection. In no instance have we observed spontaneous vascular thickening in organs other than the lungs. Of the two main factors, namely, vitamin C deficiency and diphtheria toxin, concerned in the production of the hyperplastic arteriosclerosis, the influence of the toxin appears to be the easier to evaluate from microscopic evidence. As has already been indicated, the four 0.1 M.L.D. of diphtheria toxin injected at 4-day intervals into pigs maintained on a daily vitamin ration sufficiently high to prevent obvious scorbutic symptoms (0.25 to 0.50 mg. daily) was not sufficient to produce a vascular thickening even in the more responsive pulmonary arteries. With the next higher diphtheria dosage used (0.3 M.L.D.) diffuse hyperplastic arteriosclerosis was produced regardless of the vitamin C level. About 10 days are required for the development of the sclerosis. That lack of, or decrease in, vitamin C is one of the indispensable factors in the pathogenesis of this lesion seems assured from the ease with which the lesion is produced in the guinea pig, an animal incapable of synthesizing this vitamin, in contradistinction to the practical impossibility of eliciting it in other experimental animals which can synthesize vitamin C. The smooth muscle content of even large vessels of the muscular type is small in amount and difficult to separate from the other cellular constituents. It is impossible to obtain an amount sufficient for titration of vitamin C from guinea pig vessels. In an effort to ascertain the vitamin C content of smooth muscle, vitamin C titrations were made on tissue from uterus and myomata removed at operation and on post-mortem bladder, uterus, and large and small intestinal wall freed as far as possible from mucosa and peritoneal covering.

Titrations on three human uteri and two myomata gave vitamin C values ranging between 0.1 and 0.2 mg. per gram. The intestinal values from two autopsy cases dying from infection were 0.02 and 0.07 with the higher figure for the large intestine. Determinations on bladder and uterus in the

last two cases were in the same range, about 0.03 mg. per gram. Slightly higher values were obtained from intestinal muscle of the guinea pig. The lower figures are probably explainable on vitamin depletion due to infection. In any case it would appear reasonably certain from even these few observations that the vitamin C content of normal smooth muscle is comparatively low and that it can be readily reduced in amount.

Besides the productive lesions already described, degenerative lesions in the brain and pancreas are worthy of note. The large ganglion cells of the cerebral hemispheres and cerebellum were swollen and showed varying degrees of chromatolysis, and there was frequently an associated edema.

The characteristic lesion in the pancreas was hydropic degeneration of the islets of Langerhans. Similar lesions were first produced in the guinea pig by Thomas ('24) working with B. enteritidis in our laboratories. Under low power magnification the transparency and pale color of the islets were in striking contrast to the surrounding deeply stained acinar units. On high power examination the reason for the pale color was obvious. The beta cells were enormously swollen and their cytoplasm was packed with large watery vacuoles which were outlined by the meshes of a faintly staining network. The nuclei were swollen and vesicular, although rupture of such cells was rarely seen. While the majority of the beta cells showed this hydropic degeneration, a fair number were shrunken and had a finely granular cytoplasm and a pyknotic nucleus. These two types of cellular degeneration were seen in all pigs which had received single or repeated doses of 0.3 M.L.D. or higher dosage of toxin, if the animal were killed or died during the acute intoxication. The alpha cells appeared fairly normal. Up to a certain point the hydropic degeneration was reversible because in animals which had recovered, the islets appeared normal. In pigs receiving four injections of 0.1 M.L.D. of toxin at 4-day intervals, typical hydropic degeneration was not seen, even in the animals killed shortly after the fourth dose. In

a few instances, intensity differences in staining, developing with hematoxylin and eosin, sufficed for the differentiation of the alpha and beta cells.

Various reports (Homans, '14; Allen, '22; Menten and Manning, '25; Menten, '26) have indicated a correlation between the experimentally produced hydropic degeneration in the beta cells and hyperglycemia of experimental animals. With this in mind, a series of sugar tolerance tests were made for several weeks following the 0.3 and 0.5 M.L.D. injections of toxin. The animals, after having been deprived of food for 5 to 12 hours, were given 0.5 to 1 gm. of glucose orally. Blood samples of 0.1 cc. were taken from the marginal ear vein and the reducing sugar content determined by Folin's micro method. A lowering of sugar tolerance was found with values in the range of 250 to 400 mg. per 100 cc. of blood, persisting for 2 to 3 hours. With increasing intoxication and parenchymatous damage the further lowering of the sugar tolerance became marked. With the disappearance of the symptoms and restitution of the pig to normal physiologic conditions following recovery from the toxin, there was a return to normal tolerance.

It is interesting that approximately the same amount of diphtheria toxin is required for the production of the arteriosclerosis as for the lesions in the islets of Langerhans.

## DISCUSSION

A comparison of the vascular lesions produced in rabbits and guinea pigs by injections of diphtheria toxin reveals fundamental similarities in the early stages of the pathogenesis. Thus the degenerative changes occurring in the medial smooth muscle and elastic tissue of the rabbit, as described by Klotz ('06), Bailey ('17), and Duff ('32) can be duplicated in the guinea pig. The conspicuous difference is in the connective tissue response in either animal. This morphologic difference is paralleled by the difference in vitamin C metabolism in the two animals. While the relationship of vitamin C to connective tissue is complex, the investigations of Wolbach and Howe ('26) demonstrate that in the

guinea pig an adequate vitamin C intake is indispensable for the formation and integrity of collagen. Presumably respiratory tissue metabolism is intimately adjusted to such a connective tissue relationship and herein lies the basis for the unique vascular response in the guinea pig.

Apparently variation in vitamin C metabolism is a potent factor in determining the degree of connective tissue hyperplasia in the affected arteries. Another factor concerned in this hyperplasia is the anatomic structure of the vessel, particularly the histologic constitution of the media. Degrees of degenerative change in the medial elements, together with volume and force of blood flow in the arterial bed, are further modifying factors.

A number of preliminary experiments have demonstrated that the infectious agent may vary widely since the substitution of pneumococcus, staphylococcus, B. coli, B. typhosus, B. enteritidis and green and hemolytic streptococci for diphtheria toxin yields comparable results.

It is noteworthy that the guinea pig is one of the few animals which shares with man the latter's inability to synthesize vitamin C. The production of hyperplastic arterial thickening in the guinea pig by a combination of dietary vitamin C deficiency and infection it is believed is not without significance in relation to the origin of diffuse hyperplastic arteriosclerosis in the human, despite the fact that morphologic differences exist between the two lesions. The intimal thickening characteristic of human arteriosclerosis is lacking and the selective organic distribution is not identical in the experimental form. Subsequent investigation will possibly reveal the reason for these differences. It also remains to be determined in detail how the two components, namely, vitamin C deficiency and the infectious agent, which produce their cumulative effect on the vessel wall, exert their individual influence.

Another feature brought out by our experiments which would seem to have a bearing on human pathology is the association of degeneration in the islets of Langerhans and

lowered sugar tolerance with the arteriosclerosis. The high incidence of diabetes with human arteriosclerosis has long been a subject of clinical speculation. Recently Yannet, Darrow, Goldfarb and Cary ('33, '34) have reported disturbances in blood sugar following injections of diphtheria toxin into rabbits and have interpreted the subsequent hyperglycemia as due chiefly to liver injury with resultant failure of liver balance. The fact that severe parenchymatous damage in the liver with insignificant pathology in the pancreas is sufficient to give rise to hyperglycemia in rabbits has been attested by many workers. Following injection of the B. enteritidis, its toxic products and peptone, we (Menten and Manning, '27; Menten and King, '30) have obtained such a phenomenon. Zeckwer ('25) and others (Delafield, '31, '32, '34) have shown that injection of various members of the colityphi group will produce a similar picture. Rabbits, however, are not subject to vitamin C deficiency and their tissue injury would not be complicated by disturbances arising from this source. Much of the effect on sugar tolerance observed by us may be due to liver and kidney injury but the changes produced in the beta cells of the islets of Langerhans are indicative of an additional injury to the insulin elaborating mechanism. We believe that the ease with which islet cell lesions are produced in the guinea pig is significant. The complexity of functional relationships between liver and pancreas make it necessary to continue the experiments through a longer time period before arriving at a definite conclusion. This work is being continued in order to determine whether a permanent islet damage associated with hyperglycemia and glycosuria can be produced.

SUMMARY

Guinea pigs maintained on diets deficient in vitamin C, and injected with sublethal amounts of diphtheria toxin showed diffuse hyperplastic arteriosclerosis in lungs, liver, spleen and kidneys. No vascular thickening was observed in the heart. Such animals also developed hydropic degeneration of the islets of Langerhans and an associated hyperglycemia and lowered glucose tolerance. With recovery of the animal the pancreatic lesions disappeared. The significance of these findings is discussed.

LITERATURE CITED

ALLEN, F. M. 1922 Experimental studies in diabetes; series III. The pathology of diabetes. 1. Hydropic degeneration of islands of Langerhans after partial pancreatectomy. J. Metabol. Res., vol. 1, p. 5. 4. The role of hyperglycemia in the production of hydropic degeneration of islands. Ibid., vol. 1, p. 75.

BAILEY, C. H. 1917 The production of arteriosclerosis and glomerulonephritis in the rabbit by intravenous injections of diphtheria toxin. J. Exp. Med., vol. 25, p. 109.

BESSEY, O. A., M. L. MENTEN AND C. G. KING 1934 Pathologic changes in organs of scorbutic guinea pigs. Proc. Soc. Exp. Biol. and Med., vol. 31, p. 455.

DARROW, DANIEL C., HERMAN YANNET AND M. KATHERINE CARY 1934 Physiological disturbances during experimental diphtheritic intoxication. IV. Blood electrolyte and hemoglobin concentrations. J. Clin. Invest., vol. 13, p. 553.

DELAFIELD, M. C. 1931 Changes in blood sugar and blood phosphorus in rabbits following injections of suspensions of Bact. aertrycke. J. Path. and Bact., vol. 34, p. 177.

———— 1933 Comparison of changes in blood sugar and blood phosphorus in rabbits following injection of different dead bacteria. Ibid., vol. 34, p. 53.

———— 1934 Blood sugar changes and toxic effects produced in rabbits by certain fractions derived from Bact. aertrycke. Brit. J. Exp. Med., vol. 15, p. 130.

DUFF, G. L. 1932 Medial degeneration of aorta of rabbit produced by diphtheria toxin. Arch. Path., vol. 13, p. 543.

HOMANS, JOHN 1914 Degeneration of the islands of Langerhans associated with experimental diabetes in the cat. J. Med. Res., vol. 30, p. 49.

KLOTZ, OSKAR 1906 Experimental production of arteriosclerosis. Brit. Med. J., pt. 2, p. 1767.

LINDSAY, B., AND G. MEDES 1926 Histological changes in testis of guinea pig during scurvy and inanition. Am. J. Anat., vol. 37, p. 213.

MENTEN, M. L. 1926 Soluble toxic products of enteritidis-paratyphoid B. group and their action especially in relation to blood sugar. J. Infect. Dis., vol. 38, p. 354.

MENTEN, M. L., AND C. G. KING 1930 Chemical studies on toxin produced by B. paratyphosus (aertrycke type). J. Infect. Dis., vol. 46, p. 275.

MENTEN, M. L., AND H. M. MANNING 1925 Relationship of enteritidis-paratyphoid B infections to hyperglycemia in rabbits. J. Infect. Dis., vol. 37, p. 400.

———— 1927 Hyperglycemia and hyperglycemia produced by Witte's peptone. J. Biol. Chem., vol. 72, p. 255.

MEYER, A. W., AND L. M. MCCORMICK 1928 Studies on scurvy. Stanford Univ. Press, Stanford University, Calif.

THOMAS, B. G. H. 1924 Occurrence of organisms of enteritidis-paratyphoid B group in guinea pigs. J. Infect. Dis., vol. 35, p. 407.

WOLBACH, S. B., AND P. R. HOWE 1926 Intercellular substances in experimental scorbutus. Arch. Path. and Lab. Med., vol. 1, p. 1.

YANNET, HERMAN AND DANIEL C. DARROW 1933 Physiological disturbances during experimental diphtheritic intoxication. I. Blood sugar lactic acid and non-protein and amino-acid nitrogen. J. Clin. Invest., vol. 12, p. 767.

———— 1933 II. Hepatic glycogenesis and glycogen concentration of cardiac and skeletal muscle. Ibid., vol. 12, p. 779.

YANNET, HERMAN AND WALTER GOLDFARD 1933 III. Respiratory quotients and metabolic rate. Ibid., vol. 12, p. 787.

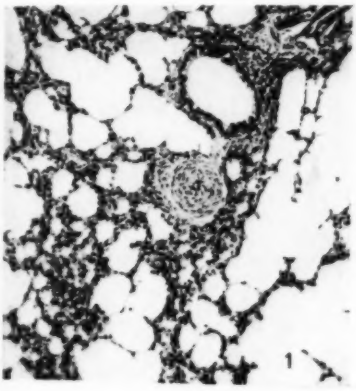
ZECKWER, I. T., AND H. GOODELL 1925 Blood sugar studies; rapid alterations in blood sugar level in rabbits as result of intravenous injections of killed bacteria of various types. J. Exp. Med., vol. 42, p. 43. Blood sugar studies; blood sugar changes in fatal bacterial anaphylaxis in rabbit. Ibid., vol. 42, p. 57.

## PLATE 1

### EXPLANATION OF FIGURES

Guinea pig (initial weight 345 gm.) fed daily on 0.3 mg. vitamin C, as orange juice, and given four injections of diphtheria toxin, each containing 0.3 M.L.D. on the sixteenth, twenty-fourth, twenty-eighth and thirtieth days. Animals died on thirtieth day.

1 Arteriosclerotic vessel of lung. × 100.
2 Arteriosclerotic vessel of lung. × 250.
3 Arteriosclerotic vessel of liver. × 60.
4 Arteriosclerotic vessel of liver. × 500.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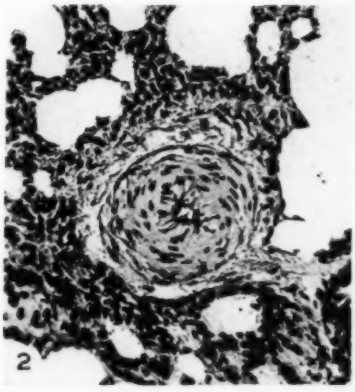
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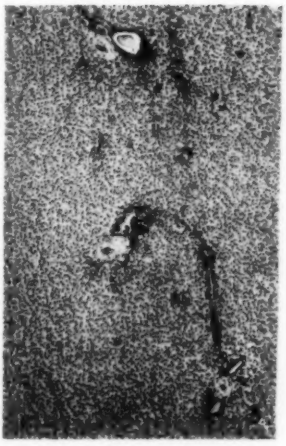

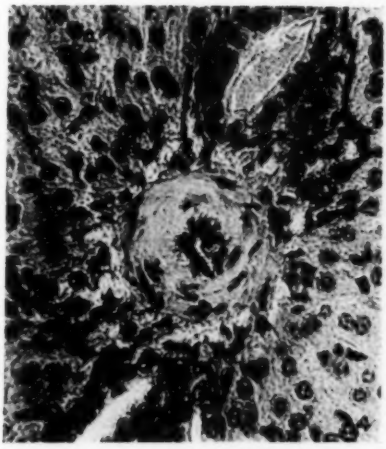

# ASCORBIC ACID (VITAMIN C) IN SPROUTED OATS [1]

RALPH BOGART AND J. S. HUGHES
*Kansas Agricultural Experiment Station, Manhattan*

TWO FIGURES

(Received for publication April 20, 1935)

During the winter months when other green feed is not available, sprouted oats has proved a satisfactory source of vitamin C for guinea pigs. After trying many ways of sprouting oats for a colony[2] of about 1000 animals, the following method has been in use the past few years. After soaking the oats in water for 5 hours, they are placed in large shallow pans, where they are well stirred and watered twice a day for 7 days. At this stage the shoots have made such development (fig. 1) that stirring is no longer practical. To continue the growth without stirring results in matting of the roots which renders the oats less desirable for feeding. Also, after the seventh day, increasing difficulty in preventing mold and bacterial development is met. For these reasons it has been found desirable to feed the oats at that stage of growth. They are fed at the rate of about 40 gm. for each animal, which amounts to approximately five hundred seedlings. Results obtained with this large colony of guinea pigs show that this method of feeding sprouted oats provides the animals' requirement of vitamin C.

Since a convenient chemical method (Bessey and King, '33) for quantitative analysis of vitamin C is known, it was decided to determine the amount of vitamin C that was actually

[1] Contribution no. 112 from the Department of Animal Husbandry. Contribution no. 189 from the Department of Chemistry.

[2] This colony is maintained by the Department of Animal Husbandry for genetic studies.

being fed. In addition, the vitamin C content of oats was determined at the different stages of sprouting from the first to the thirteenth day. Oats used for these determinations were sprouted in the same manner as was used in sprouting the oats for feeding the guinea pigs, except some of the pans were allowed to develop for 13 days. One group of pans was kept in the dark after the seventh day.

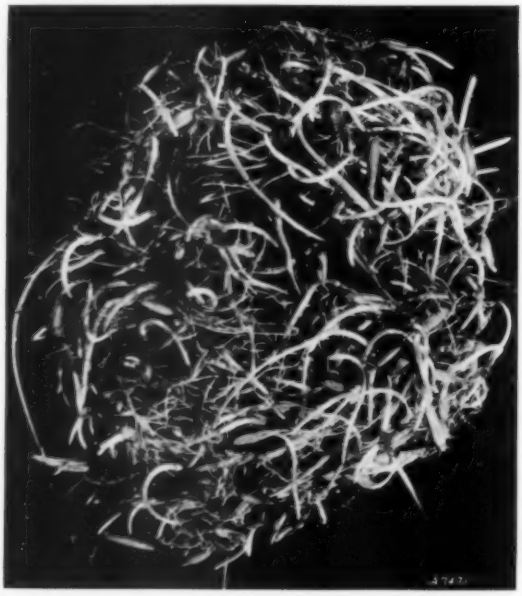

Fig. 1 Showing development of oat seedings on the seventh day of sprouting.

The ascorbic acid was determined by extracting a 5-gm. sample with 25 ml. of 3 per cent trichloracetic acid, centrifuging and titrating an aliquot of the clear filtrate with standardized 2-6-dichlorophenolindophenol.

The results which are graphically shown in figure 2 indicate a steady increase in vitamin C content up to the tenth day. After this time there seems to be a slight decline in the vitamin C content of the material under the conditions of this ex-

periment. There was very little difference in vitamin C content of the oats sprouted in the light and those in the dark, although there was considerable difference in pigmentation of the sprouts.

The vitamin C content of the various parts of the plant was determined from a sample at the seventh day. These analyses showed that over 90 per cent of the vitamin C was

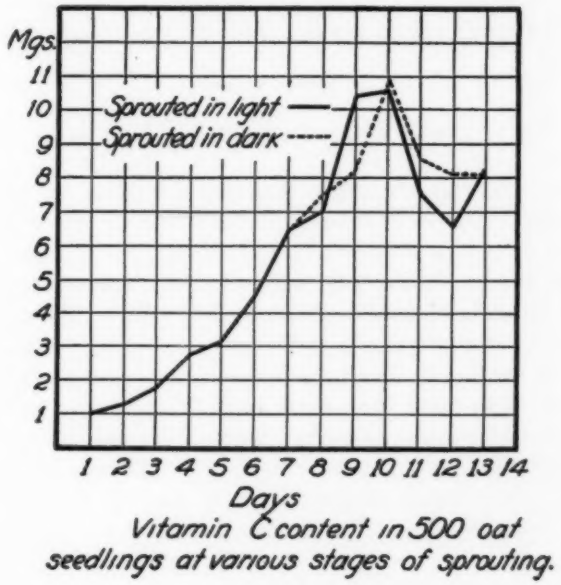


Figure 2

contained in the epicotyl. There was very little difference between the vitamin C content of the root and that of the kernel at this stage of development. If any difference, there was slightly more in the kernel.

These results indicate that each quinea pig in this colony obtains between 6 and 7 mg. of vitamin C from 40 gm. of sprouted oats a day. This would indicate that the amount of sprouted oats might be considerably reduced without endangering the animals from a deficiency of vitamin C.

## LITERATURE CITED

BESSEY, O. A. AND C. G. KING 1933 The distribution of vitamin C in plant and animal tissues, and its determination. J. Biol. Chem., vol. 103, p. 687.

# STUDIES OF CRYSTALLINE VITAMIN B

## VI. THE EFFECT OF GRADUATED DOSES ON PIGEONS

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THREE FIGURES

(Received for publication December 29, 1934)

The rat has been the test animal for the past few years in this laboratory in the development of a suitable procedure for the isolation of vitamin B($B_1$) and as a means of assay of the crystalline material. In view of the wide use of birds as B test animals in other laboratories, it was considered advisable to investigate the curative effect of the vitamin on pigeons.

Adult birds were put on a diet of autoclaved whole wheat ad lib. plus oyster shell. The wheat had been autoclaved for 4 hours at 15 pounds' steam pressure. Upon the onset of polyneuritis, usually after 3 to 4 weeks, when the birds had declined 20 to 25 per cent in weight, dilute ethyl alcohol solutions of crystalline vitamin B hydrochloride were administered orally by pipette.

Doses as low as 3 γ have always brought about some improvement and in some cases have effected cures. Four γ is generally curative and 5 γ has never failed to cure a bird with polyneuritis. Pigeons respond to a curative dose more quickly than do rats; a bird which is prostrate and shows violent head retraction is often able to stand within 2 hours and seems completely cured on the following day. The pigeons used have not been standardized as to response (Kinnersley, Peters and Reader, '28) and only a limited number have been used, which makes the data on the effect of magnitude of dose on the length of cure unsatisfactory.

Rats show a remarkable response to high levels of dosage with crystalline vitamin B (Waterman and Ammerman, '35). For comparative purposes the effect of a range of doses of the crystals on the weights of adult pigeons which had been depleted on a diet of autoclaved wheat has also been tested. The birds were fed whole wheat ad lib. plus oyster shell over a period of 2 to 3 weeks in order to set a normal weight, which ranged from 300 to 350 gm. Autoclaved wheat was then substituted for the whole wheat and after 21 days, when they had declined about 25 per cent in weight, the vitamin addendum was started. To one group 5 γ was given daily until the initial weight increase occasioned by the administration of the vitamin had given way to a further decline. The dosage was increased to 20 γ daily and kept at that amount until the birds' weights were again level. Up to this point very little weight gain had been effected, the birds being but slightly heavier than when the 5 γ dosage was started. Eighty γ daily was then fed with some further gain, but the weights leveled out at about 80 to 90 per cent of normal and additional increase to 160 γ daily was not adequate to bring the birds back to their former weight. Another depleted group was started at 10 γ daily and raised to 40 γ daily with similar results. The vitamin addendum was then stopped and after a short period when the weights had again turned down whole raw wheat was substituted for the autoclaved wheat.

Figure 1 gives the weight curves of individual birds typical of each of the two groups of four each and figure 2 shows graphically the averages of results which have been obtained. At the 5 γ dosage the pigeons were free from polyneuritic symptoms, but were unable to hold their temporary weight gains. With increasing amounts of B up to about 80 γ per day, the average weight level is successively raised. The behavior of the birds varied rather widely from individual to individual, but none was able to regain its normal weight even with the maximum amount of vitamin administered, 160 γ daily. However, in every case the change in diet to raw whole wheat always led to an immediate steady weight increase which restored the birds quickly to their normal weight.

These results constitute further evidence of the existence in wheat of a factor corresponding to that postulated by Williams and Waterman ('28) under the designation $B_3$. In

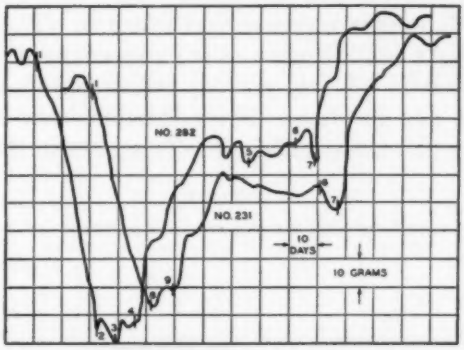


Fig. 1 Weight curves of typical pigeons on increasing vitamin B dosage. At point 1 autoclaved wheat was substituted for raw wheat. At point 2, 5 γ of the crystalline vitamin was fed daily, at point 3 the dosage was increased to 20 γ, at point 4 to 80 γ and at point 5 to 160 γ. At point 8, 10 γ was fed daily and at point 9 the dosage was raised to 40 γ. At point 6 the vitamin dosage was stopped and at point 7 the birds were again put on a diet of raw wheat.

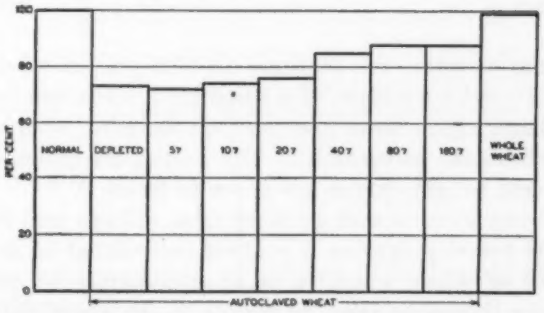


Fig. 2 Average weight level of pigeons on various dosages of crystalline vitamin B hydrochloride.

a further effort to throw light on the existence or non-existence of the so-called $B_3$, feeding tests on depleted pigeons were made with increasing amounts of raw whole wheat as addenda. Five birds at full weight were given autoclaved

wheat for 3 weeks, after which time they were about 20 per cent below top weight. The autoclaved wheat was continued ad lib. and a daily supplement of 5 gm. of whole raw wheat was added. In each of the birds under test a prompt but rather small weight increase was effected. The birds soon reached weight equilibrium and the addendum of whole wheat was increased to 7.5 gm. per day which produced a similar weight rise and leveling. The addendum was increased to 10 gm. daily which was sufficient to bring two of the birds back

TABLE 1

*Weight response of depleted pigeons to supplements of raw wheat*

| | NUMBER | | | | |
|---|---|---|---|---|---|
| | 22 | 23 | 38 | 20 | 28 |
| Weights in grams on: | | | | | |
| Raw wheat ad lib. | 330 | 350 | 325 | 345 | 340 |
| Autoclaved wheat ad lib. for 21 days. | 287 | 274 | 243 | 268 | 278 |
| Autoclaved wheat ad lib. + 5 gm. raw wheat | 304 | 298 | 266 | 285 | 284 |
| Autoclaved wheat ad lib. 7.5 gm. raw wheat | 317 | 324 | 294 | 314 | 308 |
| Autoclaved wheat ad lib. 10.0 gm. raw wheat | 326 | 351 | 313 | 321 | 326 |
| Autoclaved wheat ad lib. 12.5 gm. raw wheat | ... | ... | 325 | 324 | 333 |
| Raw wheat ad lib. | ... | ... | ... | 324 | 333 |

to normal weight. An increase to 12.5 gm. of whole wheat daily restored the weight of a third bird, while the remaining two did not attain their previous top weights[1] even when the whole wheat was given ad lib. The results are given in table 1 and typical weight curves are given in figure 3.

From our own as well as other data (Chase and Sherman, '31), one would judge the B content (calculated as the hydrochloride) of whole wheat to be approximately 5 γ per gram. Assuming this to be true, 10 gm. of whole wheat daily would be equal to 50 γ of B. When the B is administered in the form of whole wheat, 50 to 62.5 γ daily is necessary to restore

[1] A later survey of our results indicates that these top weights were 'supernormal' and due to previous liberal dosage with crystalline $B_1$ followed by a whole wheat diet. The hold-over effect on weight of large amounts of $B_1$ has been noted consistently.

the birds to normal weight levels. When the B is fed as a crystalline chemical compound even 160 γ per day is insufficient for complete weight restoration. The results reported furnish additional evidence that there is a B complex factor other than $B(B_1)$ needed for the complete nutrition of pigeons. However, regardless of the effects of $B_3$, large amounts of pure vitamin B lead to higher weight levels in adult pigeons as they do in rats.

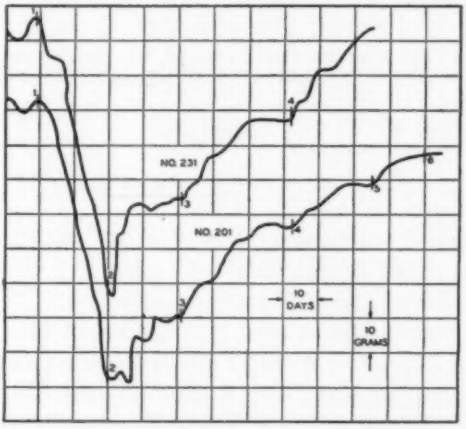


Fig. 3 Weight response of typical depleted pigeons to supplements of raw wheat. At point 1 autoclaved wheat was substituted for raw wheat. At point 2, 5 gm. of raw wheat were fed daily. At point 3 the dosage of raw wheat was increased to 7.5 gm., at point 4, to 10 gm. and at point 5 to 12.5. At point 6, whole wheat was fed ad lib.

## SUMMARY

1. The minimum dose of vitamin B hydrochloride which cures polyneuritis in pigeons is approximately 4 γ.

2. Increasing amounts of B lead to higher weight levels in previously depleted pigeons up to 80 γ per day, but even 160 γ is inadequate to bring the birds back to normal weight.

3. The significance of the results in relation to 'vitamin $B_3$' is discussed.

## LITERATURE CITED

CHASE, E. F., AND H. C. SHERMAN 1931 A quantitative study of the determination of the antineuritic vitamin B. J. Am. Chem. Soc., vol. 53, p. 3506.

KINNERSLEY, H. W., R. A. PETERS AND V. READER 1928 Antineuritic concentrates. III. The curative pigeon test. A critique. Biochem. J., vol. 22, p. 276.

WATERMAN, R. E., AND M. AMMERMAN 1935 Studies of crystalline vitamin B. V. The effect of graduated doses on growing rats. J. Nutrition, vol. 10, p. 35.

WILLIAMS, R. R., AND R. E. WATERMAN 1928 The tripartite nature of vitamin B. J. Biol. Chem., vol. 78, p. 311.

# THE RELATIONSHIP OF THE VITAMIN D INTAKE OF THE HEN TO THE ANTIRACHITIC POTENCY OF THE EGGS PRODUCED [1, 2]

N. B. GUERRANT, ELIZABETH KOHLER, J. E. HUNTER AND R. R. MURPHY

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ONE FIGURE

(Received for publication April 22, 1935)

Hess ('23) found egg yolk to be one of the few important natural sources of vitamin D. Later, Hughes, Payne, Titus, and Moore ('25) conducted an investigation in which they fed egg yolks of known source to young chicks. These authors came to the conclusion that the vitamin D content of the egg depends on the diet of the hen, as well as on the light to which she is exposed. Bethke, Kennard, and Sassaman ('27) found that eggs from range hens which received direct sunlight were ten times as potent, antirachitically, as eggs produced by hens receiving the same basal diet, but which were confined indoors where they received only light that had been filtered through glass. These authors also found that 2 per cent of cod liver oil, when added to the basal diet, induced storage of five times as much vitamin D in the egg as did the basal diet alone or the basal diet supplemented by alfalfa ad libitum.

The investigations of DeVaney, Munsell, and Titus ('33) are the most recent reported in the available literature on this subject. These investigators made biological assays on

[1] We wish to express our indebtedness to the National Oil Products Company, Harrison, New Jersey, for financial assistance in carrying out this investigation.

[2] Publication authorized by the director of the Pennsylvania Agricultural Experiment Station April 16, 1935, as technical paper no. 686.

egg yolks produced under controlled conditions, using young rats as the experimental animals. The technic employed is quite comparable to that in use at the present time. These investigators concluded that vitamin D from cod liver oil is more efficiently stored in the egg yolk than is vitamin D from an equivalent amount of viosterol, and that the vitamin D potency of egg yolk can be increased within certain limits by increasing the vitamin D intake of the hen.

Unfortunately, the data obtained in the investigations reported above cannot be compared on the basis of actual vitamin D unitage, owing to variations in the biological technic employed. It seemed desirable, therefore, that further efforts should be made toward securing additional information concerning the antirachitic potency of eggs produced under carefully controlled conditions. Since an investigation was already in progress at this institution which offered the opportunity of obtaining the eggs for such a study, it was highly desirable that an investigation be carried out at this time.

With the above facts in mind, the studies herein reported were initiated to determine the antirachitic potency of eggs produced by groups of hens which received diets varying only in their antirachitic potency.

## EXPERIMENTAL

The eggs used in these studies were obtained from groups of hens which were being used in a series of nutritional studies in the department of poultry husbandry. These birds were denied access to sunlight and were maintained on a rachitogenic basal all-mash ration which was supplemented by varying amounts of vitamin D in the form of fortified cod liver oil, viz. (8D).[3] Further information concerning the above investigation has been published elsewhere (Murphy, Hunter, and Knandel, '34).

The collection and the sampling of the eggs, and the preparation of the egg yolk samples were carried out in the following manner: The eggs were collected daily, weighed, labeled,

[3] This cod liver oil contained 100 Steenbock vitamin D units per gram, or approximately 8D potency.

and stored in a cool dark place until the end of the collection period. A complete record was kept for each bird in each of the several groups, in order that only representative eggs of each respective group might be used in making up the composite sample of yolks for biological assay. An effort was made to prepare composite samples of yolks, which represented an equal number of eggs obtained from the same number of birds in each of the respective groups.

Since only the yolks of the eggs were to be used in the biological assays, the eggs were broken and the yolks and the whites were carefully separated by hand. The selected yolks from each group of birds were weighed, thoroughly mixed, placed in sanitary cardboard containers, and stored at a temperature of $-2°$ to $-10°$F. until needed in the feeding experiments.

The antirachitic potency of these samples was determined by means of biological assay, using young rats as the experimental animals. The rats used were produced in our own stock colony, and were 21 days of age and weighed approximately 40 gm. each when placed on experiment. These animals were kept in individual metal cages which were provided with raised screen bottoms and which were housed in a darkened room, from which all daylight was excluded. At the beginning of the experiment the rats were distributed among the several groups so as to eliminate possible variations that might arise as a result of litter or sex differences. Fresh distilled water and an ample supply of the Steenbock rachitogenic diet no. 2965 was kept before each animal at all times. After having received the rachitogenic diet for a period of 18 days, the test animals were found to manifest sufficiently severe rickets for assay purposes. In each case the degree of rickets manifested was verified by means of x-ray photographs.

In the first part of the investigation, the egg yolks to be tested were incorporated directly with a known weight of the antirachitic diet. It was found in subsequent assays, however, that more consistent results could be obtained by feeding the yolk separately, in three equal portions, on the first,

third, and seventh day of the curative period; consequently the latter procedure was adopted. In this procedure, the desired quantity of yolk was weighed directly into the small glazed receptacle in which it was fed. In a few cases where the quantity was too small to be weighed conveniently, an egg yolk-basal mixture was prepared in the ratio of 1:5 or 1:10, as conditions necessitated. The amount of the mixture which contained the desired amount of yolk was then weighed in three portions and fed in the usual manner.

During the 10-day curative period, the animals were weighed on the first, third, seventh, and tenth day. On the tenth day the animals were again x-rayed, killed, and 'line tested' in the usual manner.

The biological assays consisted of three parts, which were as follows:

a. Samples of egg yolks, obtained during the fall of 1933 from nine groups of yearling hens (hatched April, 1932) that were nearing the end of their first season of egg production, were assayed biologically in the above manner.

b. In order to obtain data concerning the effect of the period of egg production on the ability of the hen to transfer antirachitic potency from the diet to the egg, a second series of biological assays was conducted. The egg yolks used in this series were obtained during the spring of 1934 from eight groups of pullets (hatched November, 1933), which were receiving dietaries similar to those reported above, but which were just beginning their first season of egg production.

c. A third series of biological assays was made using egg yolks from three groups of yearling hens. One group received an optimum level of fortified cod liver oil, and the other two groups received excessive quantities of vitamin D in the form of fortified cod liver oil and viosterol (in corn oil), respectively.

The data obtained in this investigation are presented in condensed form in the following tables and figure (tables 1 to 3 and fig. 1).

In the case of the fall series, the sampling of eggs was begun on September 5th, and continued until September 15th. Since the hens used in this series were approaching the end of a season of egg production, the egg yield was irregular. This was especially true of those groups of birds which received less than the optimum amount of cod liver oil, and which were not allowed access to range. It was thought desirable that each sample be composed of a comparatively large number of egg yolks to insure a representative sample and to have sufficient material for feeding experiments. It was planned, therefore, to use the yolks from 3 dozen eggs in each sample, except in the cases of groups IV, VI, and VIII. Samples from these groups were to contain the yolks from a larger number of eggs, as these samples were to be used in the preliminary (trial) assays. However, as may be observed from table 1, such a systematic method of sampling was impossible owing to the fact that the hens comprising groups II and III produced so few eggs.

Table 1 also presents a summary of the results of the biological assays made of the nine samples of egg yolks produced by the respective groups of hens comprising the fall series. The percentage of positive line tests was computed on the basis of the number of animals which responded normally during the test period and which gave a two plus (2+)[4] healing when line tested. When 60 per cent or more of the test animals showed a (2+) line test, after being fed a definite amount of the egg yolk, this amount of yolk was considered the critical level.

In the preliminary feeding technic, as has been previously stated, the weighed quantities of yolk were mixed with a known weight of the antirachitic diet and fed in this manner. Owing to the fact that some of the test animals used in the earlier studies died during the 10-day curative period, due in all probability to the partial decomposition of the yolk

[4] An animal was rated as showing a (2+) healing when the line test showed a narrow continuous linear deposit of calcium salts on the metaphyseal side of the epiphyseal cartilage.

during the time it remained in the feed container, exposed to the warm temperature of the laboratory, the technic was modified so as to feed the yolk in small allotments, separately of the basal diet.

TABLE 1

*Showing the results of the biological assays of the samples of egg yolks produced by groups of mature hens just before reaching the end of a period of egg production*

| GROUP NO. | PER CENT FORTIFIED COD LIVER OIL FED | NUMBER OF EGGS IN SAMPLE | WEIGHT OF EGGS IN GRAMS | PER CENT EGG YOLK | NUMBER OF RATS CONSIDERED | QUANTITY OF EGG YOLK FED GM./RAT/DAY | PER CENT POSITIVE LINE TESTS | PER CENT POSITIVE X-RAYS | CRITICAL LEVEL GM./RAT/DAY | UNITS OF VITAMIN D (STEENBOCK) PER GRAM OF EGG YOLK |
|---|---|---|---|---|---|---|---|---|---|---|
| II | 1/32 | 14 | 711 | 33.3 | 6 | 0.7 | 70 | ... | 0.7 | 0.14 |
| | | | | | 5 | 0.8 | 80 | ... | | |
| | | | | | 6 | 0.9 | 80 | ... | | |
| | | | | | 2 | 1.0 | 100 | ... | | |
| III | 1/16 | 10 | 557 | 32.1 | 2 | 0.5 | 0 | 0 | 0.7 | 0.14 |
| | | | | | 5 | 0.6 | 20 | 20 | | |
| | | | | | 5 | 0.7 | 60 | 60 | | |
| IV | 1/8 | 46 | 2459 | 32.7 | 2 | 0.1 | 0 | 0 | 0.4 | 0.25 |
| | | | | | 6 | 0.2 | 0 | 0 | | |
| | | | | | 5 | 0.3 | 20 | 0 | | |
| | | | | | 6 | 0.4 | 75 | 33 | | |
| V | 3/16 | 36 | 1999 | 31.3 | 4 | 0.2 | 25 | 0 | 0.3 | 0.33 |
| | | | | | 8 | 0.3 | 75 | 50 | | |
| | | | | | 5 | 0.4 | 60 | 60 | | |
| VI | 1/4 | 59 | 3273 | 31.3 | 2 | 0.1 | 0 | 0 | 0.2 | 0.50 |
| | | | | | 10 | 0.2 | 60 | 50 | | |
| VII | 3/8 | 36 | 2012 | 32.2 | 4 | 0.1 | 25 | 0 | 0.2 | 0.50 |
| | | | | | 4 | 0.2 | 75 | 60 | | |
| | | | | | 2 | 0.3 | 100 | 100 | | |
| VIII | 1/2 | 57 | 3113 | 32.3 | 10 | 0.1 | 60 | 40 | 0.1 | 1.00 |
| | | | | | 1 | 0.2 | 100 | ... | | |
| | | | | | 1 | 0.3 | 100 | ... | | |
| | | | | | 1 | 0.4 | 100 | ... | | |
| IX | None (range hens) | 35 | 1934 | 33.5 | 4 | 0.3 | 75 | 75 | 0.3 | 0.33 |
| | | | | | 3 | 0.4 | 100 | 100 | | |
| | | | | | 2 | 0.5 | 100 | 100 | | |
| | | | | | 4 | 0.6 | 100 | 100 | | |
| X | 1/8 (range hens) | 36 | 2011 | 31.4 | 4 | 0.025 | 50 | ... | 0.05 | 2.00 |
| | | | | | 7 | 0.05 | 71 | ... | | |
| | | | | | 2 | 0.1 | 100 | 100 | | |
| | | | | | 3 | 0.2 | 100 | 100 | | |

The results presented in the table show that, in the case of the confined groups of birds, an increase in the amount of cod liver oil fed resulted in an increase in the antirachitic

TABLE 2

*Showing the results of the biological assays of the samples of egg yolks produced by groups of pullets at the beginning of their initial period of egg production*

| GROUP NO. | PER CENT FORTIFIED COD LIVER OIL FED | NUMBER OF EGGS IN SAMPLE | WEIGHT OF EGGS IN GRAMS | PER CENT EGG YOLK | NUMBER OF RATS CONSIDERED | QUANTITY OF EGG YOLK FED GM./RAT/DAY | PER CENT POSITIVE LINE TESTS | PER CENT POSITIVE X-RAYS | CRITICAL LEVEL GM./RAT/DAY | UNITS OF VITAMIN D (STEENBOCK) PER GRAM OF EGG YOLK |
|---|---|---|---|---|---|---|---|---|---|---|
| II | 1/16 | 36 | 1402 | 27.1 | 4 | 0.7 | 25 | 0 | 0.8 | 0.12 |
| | | | | | 4 | 0.8 | 75 | 75 | | |
| | | | | | 4 | 0.9 | 100 | 75 | | |
| III | 1/8 | 36 | 1506 | 30.3 | 4 | 0.4 | 0 | 0 | 0.5 | 0.20 |
| | | | | | 4 | 0.5 | 75 | 50 | | |
| | | | | | 4 | 0.6 | 100 | 100 | | |
| IV | 3/16 | 36 | 1502 | 27.1 | 4 | 0.3 | 25 | 25 | 0.4 | 0.25 |
| | | | | | 4 | 0.4 | 75 | 50 | | |
| | | | | | 4 | 0.5 | 75 | 75 | | |
| V | 1/4 | 36 | 1464 | 27.5 | 4 | 0.3 | 0 | 0 | 0.4 | 0.25 |
| | | | | | 4 | 0.4 | 62.5 | 25 | | |
| | | | | | 4 | 0.5 | 100 | 100 | | |
| VI | 5/16 | 36 | 1556 | 26.0 | 4 | 0.2 | 0 | 0 | 0.3 | 0.33 |
| | | | | | 4 | 0.3 | 75 | 75 | | |
| | | | | | 4 | 0.4 | 100 | 100 | | |
| VII | 3/8 | 36 | 1567 | 26.2 | 4 | 0.2 | 25 | 25 | 0.3 | 0.33 |
| | | | | | 4 | 0.3 | 75 | 50 | | |
| | | | | | 4 | 0.4 | 100 | 75 | | |
| VIII | 1/2 | 36 | 1536 | 27.5 | 4 | 0.1 | 0 | 0 | 0.2 | 0.50 |
| | | | | | 4 | 0.2 | 75 | 75 | | |
| | | | | | 4 | 0.3 | 100 | 100 | | |
| IX | 1 | 36 | 1560 | 25.9 | 4 | 0.05 | 62.5 | 25 | 0.05 | 2.00 |
| | | | | | 2 | 0.075 | 75 | 50 | | |
| | | | | | 4 | 0.1 | 87.5 | 75 | | |
| | | | | | 2 | 0.2 | 100 | 75 | | |

potency of the eggs produced. When less than $\frac{1}{8}$ of 1 per cent of the fortified cod liver oil was fed (e.g., groups II and III) egg production was so poor as to make it difficult to obtain representative samples of yolks for biological assays. In fact the results indicate that $\frac{1}{16}$ of 1 per cent of the cod liver

oil was not better than $\frac{1}{32}$ of 1 per cent, in increasing the antirachitic potency of the egg yolks. It was interesting to note, however, that range hens which received no cod liver oil, but had access to sunlight (group IX) produced eggs equal in antirachitic potency to those produced by confined hens receiving the same diet supplemented with $\frac{5}{16}$ of 1 per cent of cod liver oil (group V). An addition of $\frac{1}{8}$ of 1 per cent

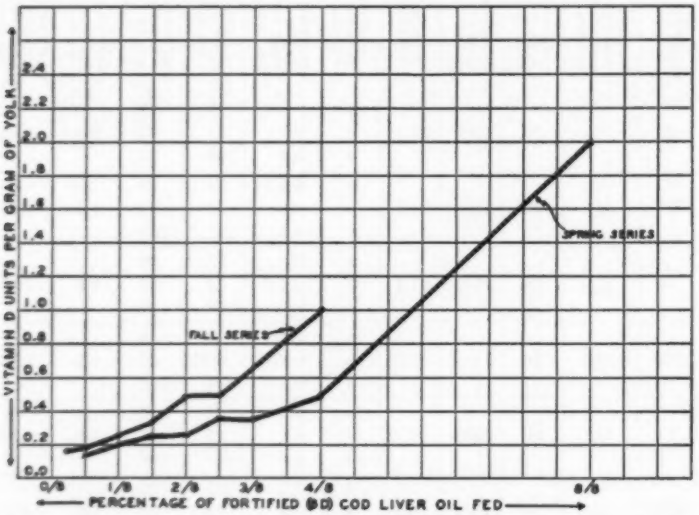


Fig. 1 Showing graphically the relationship between the percentage of fortified cod liver oil (8D) fed and the antirachitic potency of the eggs produced by the various groups of hens used in this investigation.

of cod liver oil to the diet of the range hens resulted in the production of eggs having approximately six times as much antirachitic potency as those produced by range hens receiving no cod liver oil (groups IX and X).

The relationship between the amounts of cod liver oil fed and the antirachitic potency of the eggs produced is presented graphically in figure 1.

Table 2 contains a summary of the data obtained in the second series of experiments. Since a systematic method of

sampling was possible in the case of this series, the samples consisted of two eggs from each of eighteen hens which were representative of the respective groups. It was necessary, however, to extend the collecting period for group II, 2 additional days, in order to obtain the desired number of eggs.

The vitamin D assay data found in this table were compiled in a manner similar to that described for table 1. Here, as in the first series, an increase in the amount of cod liver oil led to the production of eggs of higher antirachitic potency.

TABLE 3

*Showing the results of the biological assays of the samples of egg yolks produced by the three groups of hens, two of which received excessive doses of vitamin D, as fortified cod liver oil and viosterol, respectively*

| GROUP NO. | PER CENT FORTIFIED COD LIVER OIL FED | NUMBER OF EGGS IN SAMPLE | WEIGHT OF EGGS IN GRAMS | PER CENT EGG YOLK | NUMBER OF RATS CONSIDERED | QUANTITY OF EGG YOLK FED GM./RAT/DAY | PER CENT POSITIVE LINE TESTS | PER CENT POSITIVE X-RAYS | CRITICAL LEVEL GM./RAT/DAY | UNITS OF VITAMIN D (STEENBOCK) PER GRAM OF EGG YOLK |
|---|---|---|---|---|---|---|---|---|---|---|
| I (control) | ¼ % SD oil | 16 | 917 | 31.5 | 4 | 0.1 | 75 | 60 | 0.1 | 1.00 |
| | | | | | 8 | 0.2 | 100 | 62 | | |
| | | | | | 8 | 0.3 | 88 | 88 | | |
| | | | | | 4 | 0.4 | 100 | 100 | | |
| II (SD cod liver oil) | 2 % SD oil | 24 | 1299 | 30.8 | 4 | 0.015 | 25 | 25 | 0.025 | 4.00 |
| | | | | | 7 | 0.025 | 70 | 57 | | |
| | | | | | 4 | 0.035 | 100 | 100 | | |
| | | | | | 4 | 0.05 | 100 | 100 | | |
| | | | | | 4 | 0.1 | 100 | 100 | | |
| III (viosterol) | 2 % SD viosterol | 9 | 497 | 32.1 | 4 | 0.015 | 0 | 0 | 0.025 to 0.05 | 2.00 to 4.00 |
| | | | | | 8 | 0.025 | 37.5 | 25 | | |
| | | | | | 4 | 0.05 | 100 | 100 | | |
| | | | | | 4 | 0.1 | 100 | 100 | | |

The results of the biological assays were, in general, quite comparable to the results obtained through the fall series of assays. It is to be noted, however, that for a given level of cod liver oil the pullets at the beginning of a season of egg production produced eggs which were slightly less potent, antirachitically, than were eggs produced by yearling hens at the end of a season of production. But in this particular investigation, it appears that the number of eggs produced

might have been a more important factor in determining this difference in potency than the stage of egg production.

The relationship between the amount of cod liver oil fed and the antirachitic potency of the eggs produced by the various groups of pullets comprising the spring series is also presented graphically in figure 1.

The third phase of the investigation was concerned with the effect of feeding excessive quantities of vitamin D on the antirachitic potency of the eggs produced. Summaries of the collecting and the sampling of the eggs used in this series of assays and of the results of the biological assays are presented in table 3. The data given in this table were compiled in a manner similar to that described for the other series.

Considerable difficulty was experienced in obtaining sufficient eggs to comprise a representative sample from the group of hens which received the high intake of viosterol. This difficulty, however, was not encountered with the group of hens that received an equivalent amount of vitamin D in the form of fortified cod liver oil.

From table 3 it may be observed that when the antirachitic supplement was increased eightfold, by the addition of fortified cod liver oil, there resulted a fourfold increase in the antirachitic potency of the eggs produced (groups I and II). When the increased antirachitic supplement was fed in the form of viosterol (in corn oil), the antirachitic potency of the eggs produced was somewhat less than when an equivalent unitage of cod liver oil was fed.

Since the initiation of this investigation, there have appeared in print the publications of Branion ('34), Branion, Drake, and Tisdall ('34, '35), which likewise concern the vitamin D content of eggs. While the data herein presented do not permit a direct comparison with those presented by the above investigators (due to differences in methods of technic and in manner of presenting results), it appears that the data do indicate a somewhat lower antirachitic potency than might have been reasonably expected from the findings of the above investigators.

SUMMARY

A study has been made of the effect of the vitamin D intake of the hen on the antirachitic potency of the eggs produced. As a result of this investigation, the following conclusions are drawn:

1. The antirachitic potency of egg yolk depends on the antirachitic intake of the hen producing it.

2. The ability of the hen to transfer the antirachitic factor or factors from her diet to the egg appears to be limited.

3. When the hen's diet is supplemented with one-fourth per cent of fortified (8D) cod liver oil (which Murphy, Hunter, and Knandel ('34) found to be the optimum level when the birds were confined and denied access to direct sunlight), 1 gm. of the yolk from the eggs produced contained approximately 0.5 Steenbock unit. On this basis, an egg yolk weighing 15 gm. would contain 7.5 Steenbock units of vitamin D.

4. Yearling hens, nearing the end of a period of egg production, produced egg yolks that were more potent (antirachitically) than did pullets at the beginning of their initial period of egg production. It is suggested that this difference in potency might be due to the rate of egg production and not to the period of production.

5. At the particular level studied, the antirachitic potency of cod liver oil appeared to be more efficiently transferred from the diet to the egg yolk than was an equal unitage of viosterol.

## LITERATURE CITED

BETHKE, R. M., D. C. KENNARD AND H. L. SASSAMAN 1927 The fat soluble vitamin content of hen's egg yolk as affected by the ration and the management of the layers. J. Biol. Chem., vol. 72, p. 695.

BRANION, H. D. 1934 The vitamin D content of eggs. Canadian Public Health J., April.

BRANION, H. D., T. G. H. DRAKE AND F. F. TISDALL 1934 Vitamin D content of egg yolk. The U. S. Egg and Poultry Magazine, vol. XL, July, p. 20; August, p. 22; September, p. 22.

———— 1935 The vitamin D content of egg yolk. The Canadian Medical Association J., vol. 32, p. 9.

DEVANEY, G. M., H. E. MUNSELL AND H. W. TITUS 1933 The effect of sources of vitamin D on storage of the antirachitic factor in the egg. Poultry Science, vol. 12, p. 215.

HESS, A. F. 1923 The therapeutic value of egg yolk in rickets. Proc. Soc. for Exp. Biol. and Med., vol. 20, p. 269.

HUGHES, J. S., L. F. PAYNE, R. W. TITUS AND J. M. MOORE 1925 The relation between the amount of ultra-violet light received by hens and the amount of the antirachitic vitamin in the eggs produced. J. Biol. Chem., vol. 87, p. 421.

MURPHY, R. R., J. E. HUNTER AND H. C. KNANDEL 1934 The vitamin D requirements of growing chicks and laying hens. Agr. Exp. Sta. Bull. no. 303 of the Pennsylvania State College.

# QUANTITATIVE EXPERIMENTS ON THE OCCURRENCE OF VITAMIN B IN ORGANS

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TWO FIGURES

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Before the differentiation of vitamin B into its two factors, $B(B_1)$ and $G(B_2)$, investigations had been made to show the antineuritic and the growth-promoting effects of different organs of the body. Cooper ('12) showed that smaller amounts of sheep's brain and ox-heart were necessary to protect against polyneuritis than of beef muscle. Later Cooper ('14) concentrated, from cardiac muscle, a substance which would cure polyneuritis and Funk ('12) concentrated a similar substance from ox-brain. Eddy ('17) improved the growth of children and of rats suffering from marasmus by giving them an extract of sheep's pancreas.

Osborne and Mendel ('18) showed that 19 per cent dried pig's cardiac muscle, 22 per cent kidney, 32 per cent brain or 10 per cent liver, as the sole source of protein and water-soluble vitamin B in the diet, induced normal growth but that 5 per cent liver failed to do so. Drummond ('18) found that the protein-free extract of whole fish, as the only source of vitamin B, produced good growth, and Chick and Hume ('17) proved that the ova of fish contained the antineuritic vitamin. Hoagland and Lee ('24) discovered that dried hen's liver was of lower value for preventing polyneuritis than the liver from ox, sheep, or hog.

Many workers have reported that skeletal muscle is a poor source of vitamin B. Grijns ('01) noticed that raw meat protected fowls from polyneuritis while sterilized meat did not. Osborne and Mendel ('17) reported that 20 per cent dried lean beef in the diet as sole source of protein and of vitamin B, was not suitable for growth, that meat extract supported slight growth but that meat residue was unable to maintain weight. Hoagland ('29) found lean pork a rich source of vitamin B.

Osborne and Mendel ('23), by feeding liver from rats on a high and on a low vitamin B diet, showed the effect of diet on the vitamin content of the tissues. One hundred milligrams of dried liver daily from animals on an adequate diet, supported almost normal growth while twice this amount from animals deprived of vitamin B did not. Recently Westenbrink ('32) investigated the vitamin B content of the organs of white rats on diets of high and low vitamin B content and showed a rapid loss in the organs of the animals on deprivation of this vitamin.

Since the investigations of some of these workers indicated a storage of vitamin B in certain of the tissues of the rat, and the influence of diet thereon, it was the purpose of this investigation to determine more quantitatively the storage of vitamin B in the body of animals of known dietary history, and the influence of diet on that storage.

## EXPERIMENTAL

*General method.* The rat growth method for the determination of vitamin B developed by Chase and Sherman ('31) in this laboratory was used. The tissues to be tested were fed to rats which had been depleted of their stores of vitamin B and were receiving a diet free from vitamin B but adequate in other respects. These tissues served as the only source of this vitamin.

*Rats used for feeding.* The rats, whose tissues were fed as sources of vitamin B were healthy young adults, reared on diet B of this laboratory which consists of whole milk powder,

one-third by weight, whole wheat, two-thirds by weight, and salt to the extent of 2 per cent of the weight of the wheat. Males and females were used alternately, care being taken not to feed the tissues of females whose bodies had been depleted by the recent bearing and suckling of young.

The animals were chloroformed as they were needed and bled by cutting the jugular vein and artery. The tissues to be tested were removed and kept on ice in glass stoppered bottles while the separate amounts were being weighed. The portions were then fed at once. These portions were weighed on small watch glasses and fed three times a week.

*Distribution of vitamin B in the body of the rat.* Quantitative determinations were made of the relative vitamin B content of muscle, liver, kidney, heart, brain, lung, spleen, and blood of adult rats which had been fed as described above. In the case of liver and muscle, young rats of the same litter were fed amounts that would give different rates of growth but the other tissues were fed at one level only. The portions of kidney, lung, brain, and spleen chosen for feeding were the largest average amounts that could be obtained from the animals. Figure 1 shows the average gains of these animals.

From the results obtained we can conclude that, in rats fed as here described, liver is gram for gram ten times as rich a source of vitamin B as is muscle. Kidney is about one-half and brain is about one-third as rich per gram as liver. Heart is almost as rich as liver and nearly ten times as rich as muscle. Spleen, lung, and blood contain only traces of the vitamin.

## INFLUENCE OF DIET ON VITAMIN B CONTENT OF TISSUES

*A comparison of the tissues of rats on a diet rich in vitamin B with tissues from rats on a diet lacking vitamin B.* The amounts of vitamin B in the different tissues of rats reared on a normal diet (diet B) were determined by feeding the tissues to experimental animals. Using the results so obtained as standards, a comparison was made with tissues from rats on a

vitamin B free diet and with rats receiving a diet rich in vitamin B.

Young growing rats, reared to 4 months of age on diet B, were divided into two groups. One group was given a diet lacking vitamin B while another group was continued on diet B, to which had been added 2 per cent of brewers' yeast to augment the vitamin B content. When the rats, which received no vitamin B, had been on the vitamin B free diet between 4 and 5 weeks, they showed typical symptoms of vitamin B deficiency. All were emaciated, weak, had a hunched-

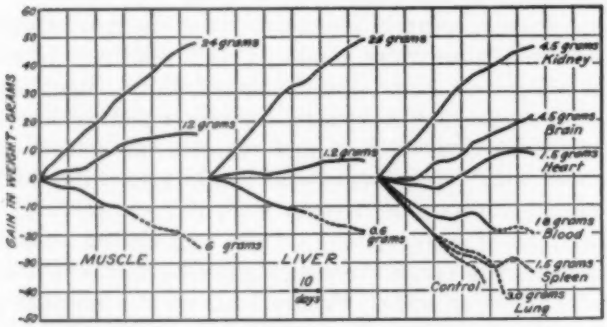


Fig. 1 Average gain curves of rats receiving a vitamin B free diet plus different amounts of tissue per week from animals reared on diet B. Broken line indicates point at which one or more rats died.

back appearance and appeared very torpid. Some had developed polyneuritis. At this time their tissues were fed to the experimental animals. At the same time, the tissues of the group which had received for the same length of time, extra vitamin B in the form of yeast added to diet B, were also fed to the experimental animals. Figure 2 shows the average gains of these animals.

The precautions stated previously were observed. The portions, except kidney, were fed at the same level as before. It was not possible to obtain as large an amount of kidney from the animals depleted of vitamin B as from normal animals.

The results obtained by feeding experimental animals the tissues of rats kept for 1 month on a diet free from vitamin B showed that there was too little vitamin B to measure in

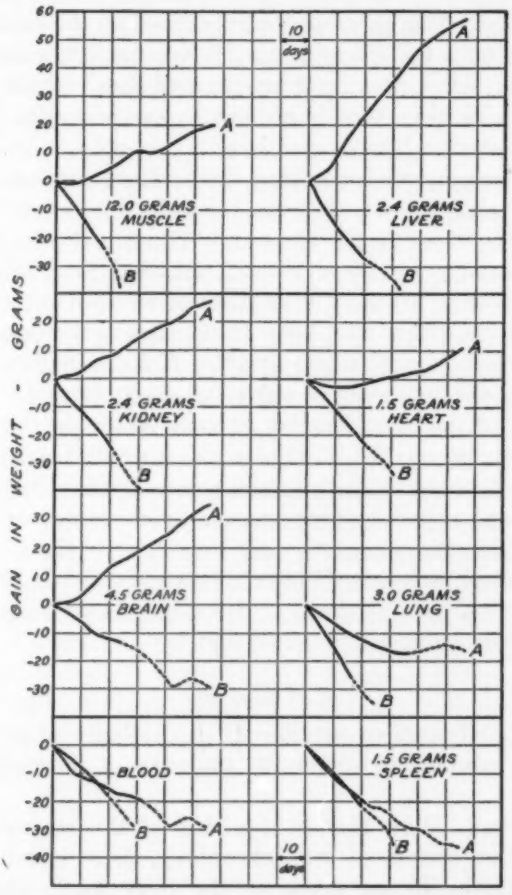


Fig. 2 A, Average gain curves of rats receiving a vitamin B free diet plus different amounts of tissue per week from animals reared on diet B plus 2 per cent yeast. B, Average gains curves of rats receiving a vitamin B free diet plus different amounts of tissue per week from animals reared on a vitamin B free diet. Broken line indicates point at which one or more rats died.

any of the tissues except brain. The animals which received brain as their supply of the vitamin did not maintain their weight but survived an average of 17.8 days longer than did the negative controls. Two animals developed severe polyneuritis. The gains and survivals of all the other experimental animals fed tissue from the rats receiving the vitamin B free diet for 1 month, approximated those of the negative controls indicating, that in all tissues except brain, there was too little vitamin B to be measured.

In the group which received tissue from the animals kept on the vitamin B rich diet for 1 month, there is evidence of a decided increase in the amount of vitamin B stored in some of the tissues. Of these tissues, muscle, liver, kidney, and brain appear to increase their store of vitamin B in about the same relative proportion, since in the case of the experimental animals receiving the tissue of animals fed a diet rich in vitamin B the average growth was from about 10 to 15 gm. more than that of the experimental animals receiving the tissue of animals reared on diet B. There was a small increase of vitamin B in the lung of the animals receiving the vitamin B rich diet. Heart, spleen and blood seem to store no extra vitamin B since the growth curves and survivals of the animals receiving heart, spleen and blood from animals reared on diet B plus yeast parallel those of the animals receiving the same tissues from animals reared on diet B.

## SUMMARY

The distribution of vitamin B in the body of rats reared on diets containing different amounts of vitamin B was studied.

The results show that in the body of the rat reared on a normal diet, diet B of this laboratory, there is ten times as much vitamin B per gram in the liver as in the muscle. Kidney is about one-half and brain is about one-third as rich per gram as liver. Heart is almost as rich as liver and nearly ten times as rich per gram as muscle. Blood, spleen, and lung, in the amounts fed, seemed to contain only traces of the vitamin.

By comparing the growth of experimental animals which had been given tissue from animals fed for 1 month on a diet lacking vitamin B with the growth of experimental animals receiving tissue from animals kept for 1 month on a normal diet (diet B) to which had been added 2 per cent of brewers' yeast to augment the vitamin B content, it was found that the former animals, except for those receiving brain, lived no longer on an average than did the negative controls. This showed either a complete lack of vitamin B in the tissues of the depleted animals or too small an amount to be detected by this method. The animals receiving the brain tissues lived longer than did the negative controls, some even surviving the experimental period, but all lost weight rapidly. The experimental animals receiving the tissues of rats fed for 1 month on a diet containing extra yeast showed increased storage of vitamin B in some of their tissues. In the case of the experimental animals receiving muscle, liver, kidney and brain, there was an increase in growth of from 10 to 15 gm. over that made by the animals receiving tissue of rats reared on the normal diet. The experimental animals receiving lung lived an average of 12 days longer than did the experimental animals receiving the lung tissue of animals reared on the normal diet. Heart, spleen and blood showed no evidence of extra storage of vitamin B when the amount of vitamin B in the diet was increased.

It has been shown, therefore, by these experiments that the amount of vitamin B in the body of the rat may be changed within certain limits by varying the amount of vitamin B in the diet.

## LITERATURE CITED

CHASE, E. F. AND H. C. SHERMAN 1931 A quantitative study of the determination of the antineuritic vitamin B. J. Am. Chem. Soc., vol. 53, p. 3506.

CHICK, H. AND M. HUME 1917 The distribution among food stuffs (especially those suitable for the rationing of armies) of the substance required for the prevention of (a) beriberi and (b) scurvy. J. Roy. Army Med. Corps, vol. 29, p. 121.

COOPER, E. A. 1912 On the protective and curative properties of certain food stuffs against polyneuritis induced in birds by a diet of polished rice. J. Hygeine, vol. 12, p. 436.

———— 1914 The relations of vitamine to lipoids. I. Preparation of an antineuritic substance from cardiac muscle. Biochem. J., vol. 8, p. 348.

DRUMMOND, J. C. 1918 The nutritive value of certain fish. J. Physiol., vol. 52, p. 95.

EDDY, W. H. AND J. G. ROPER 1917 The use of pancreatic vitamin in cases of marasmus. Am. J. Dis. Child., vol. 14, p. 189.

FUNK, C. 1912 The preparation from yeast and certain food stuffs of the substance the deficiency of which in diet occasions polyneuritis in birds. J. Physiol., vol. 45, p. 75.

GRIJNS, G. 1901 Over polyneuritis gallinarum. Genes. Tijds. Ned., Ind., vol. 41, p. 3.

HOAGLAND, R. 1929 Antineuritic and water-soluble B vitamins in beef and pork. J. Agr. Research., vol. 38, p. 431.

HOAGLAND, R. AND A. R. LEE 1924 Antineuritic vitamin in poultry flesh and eggs. J. Agr. Research, vol. 28, p. 461.

OSBORNE, T. B. AND L. B. MENDEL 1917 Nutritive factors in animal tissues. J. Biol. Chem., vol. 32, p. 309.

———— 1918 Nutritive factors in animal tissues. II. J. Biol. Chem., vol. 34, p. 17.

———— 1923 The effect of diet on the content of vitamine B in the liver. J. Biol. Chem., vol. 58, p. 363.

WESTENBRINK, H. G. K. 1932 Über den Gehalt an Vitamin-$B_1$ der Organe von weissen Ratten bei normaler und Vitamin-$B_1$-freier Ernahrung. Arch. Néerland de Physiol., Bd. 17, S. 560.

# THE EFFECT OF DINITROPHENOL ON CALCIUM AND PHOSPHORUS METABOLISM

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Increased excretion of calcium and phosphorus characteristically accompanies the elevation of the basal metabolic rate due to hyperthyroidism but does not occur in conditions of increased metabolism due to fever or leukemia (Aub et al., '29). It has been suggested that the intrinsic metabolic rate of bone tissue may be quite independent of changes in the metabolic rate of the other tissues of the body and that the effect of the thyroid hormones on calcium metabolism is due to a specific augmentation of osteoclastic processes (Albright, Bauer and Aub, '31). The work here reported was undertaken to ascertain whether dinitrophenol might possess the property of increasing bone metabolism in addition to its demonstrated action in elevating the metabolism of entire animals (Magne, Mayer and Plantefol, '32; Tainter and Cutting, '33), surviving viscera and tissues (De Meio, Martin and Field, '34; McCord, '34) and yeasts (Field, Martin and Field, '34). Conclusions regarding bone metabolism are based on the assumption that significant changes in the rate of bone formation or destruction will probably be reflected in appreciable changes in the excretion of the chief mineral constituents of bone, calcium and phosphorus.

## METHODS

The subjects of these studies were three female patients with atrophic arthritis on the medical wards of the New Haven Hospital. In every case the arthritis was of at least

10 years duration and the clinical course had been essentially stationary for some time prior to the experimental period.[1] Although x-rays revealed varying degrees of bone rarefaction and overgrowth, depending on the site and duration of the joint disease in each individual case, there were no abnormalities of calcium and phosphorus metabolism apparent in the balance studies.

The general plan of the balance experiments and the technics of the collection, preparation and analyses of the excreta have been described in a previous communication (Robbins and Kydd, '35). Essentially the same diet, identical for each day of the experiments, was given to each subject. Except in the case of subject 2, who occasionally refused some food because of gastric distress, the diets were eaten completely. A 3-day fore-period to allow adjustment to the experimental diet preceded the collection of excreta in each case.

## RESULTS

The results of the experiments are recorded in the table. In the interests of brevity only the average figures for the balances before and during the administration of dinitrophenol are presented. In case 1 the balances of calcium and phosphorus were definitely negative, while in cases 2 and 3 an identical diet resulted in positive balances. The balance of nitrogen was negative in case 1, positive in case 2 and in equilibrium in case 3. In none of the cases did the excretion of nitrogen, calcium or phosphorus change significantly when the basal metabolic rate was increased 37 per cent by the administration of dinitrophenol. The excretion of ammonia and the values for titratable acidity minus $CO_2$ in the urine of case 1 remained unaffected by the increase in oxidative metabolism. At the beginning of the administration of dinitrophenol to case 1, there was a mild diuresis with a slight increase in urine chlorides, but thereafter the chloride excretion returned to the level observed in the control periods. A similar diuresis did not occur in the other two cases.

[1] The report of the clinical response of these and other patients with chronic atrophic arthritis to the administration of dinitrophenol is reserved for future publication.

*Nitrogen, calcium and phosphorus metabolism per 3-day period (dinitrophenol periods are in italics)*

| CASE NO. | PERIODS | DATES | NITROGEN | | | CALCIUM | | | | PHOSPHORUS | | | | | BASAL METABOLISM | WEIGHT |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Intake | Output | Balance | Intake | Urine | Stool | Balance | Intake | Urine | Stool | Balance | Date | | |
| | | *1934* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | | *per cent* | *kilos* |
| 1 | I and II | 8/22–8/28 | 31.5 | 36.2 | — 4.7 | 1.89 | 0.70 | 1.56 | — 0.37 | 3.18 | 2.32 | 1.26 | — 0.40 | 8/27 | — 12 | 55.4 |
| | *III and IV* | *8/28–9/3* | *31.5* | *34.6* | *— 3.1* | *1.89* | *0.76* | *1.57* | *— 0.44* | *3.18* | *2.32* | *1.16* | *— 0.30* | *9/4* | *+ 25* | *54.7* |
| 2 | I and II | 5/29–6/4 | 30.7 | 26.2 | + 4.5 | 1.80 | 0.25 | 1.45 | + 0.10 | 3.04 | 1.60 | 1.11 | + 0.33 | 6/4 | + 4 | 56.9 |
| | *III and IV* | *6/4–6/10* | *30.8* | *25.2* | *+5.6* | *1.84* | *0.20* | *1.39* | *+ 0.25* | *2.99* | *1.38* | *1.04* | *+ 0.57* | *6/11* | *+ 42* | *56.6* |
| 3 | I–III | 1/10–1/19 | 31.5 | 36.5 | — 5.0 | 1.89 | 1.86[1] | | + 0.03 | 3.18 | 2.97[1] | | + 0.21 | 1/3 | — 15 | 77.2 |
| | *IV–VII* | *1/19–1/31* | *31.5* | *31.9* | *— 0.4* | *1.89* | *1.79[1]* | | *+ 0.10* | *3.18* | *3.00[1]* | | *+ 0.18* | *2/3* | *+ 22* | *76.6* |

[1] Total output in stool and urine mixture.

Examination of the blood serum at frequent intervals during the individual experiments revealed no changes in the normal concentrations of calcium, phosphorus and proteins of the serum.

## DISCUSSION

Since each subject served as her own control, speculations concerning differences in the individual response to an identical diet are not germane to the general observation that dinitrophenol in doses sufficient to elevate the basal metabolic rate 37 per cent above the normal does not appear to affect the rate of excretion of calcium and phosphorus. Whether or not the actual respiratory exchange of the cellular elements of bone is increased by dinitrophenol is beyond the scope of metabolic balance studies but certainly there is no significant change in the rate of deposition or resorption of bone salts. According to the theory (Albright, Bauer and Aub, '31) which attributes osteohalisteresis in hyperthyroidism to a specific factor in the thyroid hormone the negative results with dinitrophenol could have been predicted.

The failure of dinitrophenol to influence nitrogen excretion is in agreement with the results of the animal experiments of Magne, Mayer and Plantefol ('32) and the clinical studies of Cutting and Tainter ('33). Although from the data here presented it is not possible to reach any final conclusion regarding the metabolic mixture during the dinitrophenol periods, it would appear that protein combustion was not increased. This is especially evident in case 1 where the negative nitrogen balance in the control periods indicates an inadequate supply of exogenous protein for the metabolic mixture even when the basal metabolic rate was 12 per cent below normal. The acceleration of metabolism to 25 per cent above normal in a period of 6 days did not increase the rate of depletion of the tissue proteins; on the contrary, there was, if anything, a tendency for the contribution of body proteins to the metabolic mixture to diminish.

## SUMMARY AND CONCLUSIONS

Calcium, phosphorus and nitrogen balances in three subjects before and during administration of dinitrophenol are reported. An increase of 37 per cent in the basal metabolic rate is accompanied by no significant change in the excretion of these elements. The excretion of ammonia and the titratable acidity minus $CO_2$ in the urine of one subject was unaltered by the increase in oxidative metabolism due to dinitrophenol. The studies offer further evidence that increased metabolism of the body as a whole is not necessarily shared by the skeleton, at least in so far as changes in the intrinsic metabolism of bones are measurable by alterations in the total exchanges of the bone salts.

## LITERATURE CITED

ALBRIGHT, F., W. BAUER AND J. C. AUB 1931 Studies of calcium and phosphorus metabolism. VIII. The influence of the thyroid gland and the parathyroid hormone upon the total acid-base metabolism. J. Clin. Invest., vol. 10, p. 187.

AUB, J. C., W. BAUER, C. HEATH AND M. ROPES 1929 Studies of calcium and phosphorus metabolism. III. The effects of the thyroid hormone and thyroid disease. J. Clin. Invest., vol. 7, p. 97.

CUTTING, W. C., AND M. L. TAINTER 1933 Metabolic actions of dinitrophenol with the use of balanced and unbalanced diets. J. Am. Med. Assn., vol. 101, p. 2099.

DE MEIO, R. H., AND BARRON E. S. GUZMAN 1934 Effect of 1-2-4 D.N.P. on cellular respiration. Proc. Soc. Exp. Biol. Med., vol. 32, p. 36.

FIELD, J., 2ND, A. W. MARTIN AND S. M. FIELD 1934 Effect of 2-4 dinitrophenol on inhibition of yeast respiration by cyanide. Proc. Soc. Exp. Biol. Med., vol. 31, p. 997.

MAGNE, H. A., A. MAYER AND L. PLANTEFOL 1932 Études sur l'action du dinitrophénol 1-2-4 (thermol). II. Quelques retentissements de l'intoxication mortelle par le dinitrophénol 1-2-4 sur les phenomenes généraux de la nutrition. Ann. de physiol. physicochim. biol., T. 8, p. 51.

MCCORD, W. M. 1934 The effect of 2-4 dinitrophenol on the oxygen uptake of kidney and liver tissue of the rat. Am. J. Physiol., vol. 109, p. 232.

ROBBINS, C. L.,AND D. M. KYDD 1935 A note on the metabolic criteria of hyperparathyroidism. J. Clin. Invest., vol. 14, p. 220.

TAINTER, M. L., AND W. C. CUTTING 1933 Febrile, respiratory and some other actions of dinitrophenol. J. Pharm. Exp. Therap., vol. 48, p. 410.

# SUPPLEMENTARY VALUES OF ANIMAL PROTEIN CONCENTRATES IN CHICK RATIONS

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TWO FIGURES

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An extensive variation in the nutritive value of animal protein concentrates, when employed as supplementary sources of protein in chick rations, has been convincingly demonstrated by Prange, Carrick and Hauge ('28, '28 a, '28 b). Titus, McNally and Hillberg ('30), Allardyce, Henderson and Asmundson ('33), Johnson and Brazie ('34), Record, Bethke and Wilder ('34) and Record, Bethke, Wilder and Kennard ('34).

In experiments designed to investigate further this variation in nutritive value, similar results have been obtained which are the basis of the present report.

## PROCEDURE

The plan of research consisted in supplementing an otherwise adequate basal ration for chicks with equal levels of crude protein from various animal protein concentrates, primarily meat scraps, tankages, cracklings and similar by-products of the meat packing industry. In addition, a sample of beef, prepared in the laboratory by vacuum drying at 100°C. and then extracting most of the fat with petroleum ether, was also fed as representing the ideal meat protein concentrate. Analyses and brief descriptions of these materials are given in table 1.

In the first trial, the mixed feeds were composed of ground wheat 23 parts, ground yellow corn 56 parts, dried brewer's yeast 2 parts, salt 1 part, biologically tested sardine oil 1 part and the protein concentrate in amount sufficient to supply 6 per cent of crude animal protein. The total protein level was approximately 16 per cent. In trial II, the feeds were composed of the same ingredients in the same proportions, except that the crude animal protein level was raised to 8 per cent, the total protein level was raised to 18 per cent and the amount of yeast was increased to 3 per cent. Bone ash was added, where necessary, to bring the feeds to the same ash content. In trial III, the feeds were mixed as in trial II, except that the level of crude animal protein was 7 per cent and starch was used in place of some of the ground wheat in order to lower the vegetable protein to 9 per cent. The total protein level was 16 per cent. The mixtures were checked by analyses and adjustments made where necessary. The feeds were uniform and adequate in all respects, except for the variation in animal protein quality.

Day-old single comb White Leghorn chicks were placed in electrically heated battery brooders and given the various mixed rations and water ad. lib. The weights of the chicks and the weights of the feed consumed were determined at intervals of 2 weeks for a period of 6 weeks. The temperature of the brooders was adjusted to 95°F. for the first week and then lowered 5° per week for the remainder of the period. Special precautions were taken to avoid wastage of feed in trials II and III. Results of these feeding trials are given in table 2.

The gain per unit of feed consumed, as given in table 2, was calculated for the last 2 weeks of the growing period, rather than for the entire period, since about one-half of the total growth and total feed consumption occurred during this interval and mortality was at a minimum. Such mortality as occurred was not attributable to any specific cause and the chicks which died were considered constitutional weaklings that should not be included in growth summaries. The

feed consumed by chicks dying during the last 2 weeks was corrected for on the assumption that such chicks ate at an average rate until 1 day preceding death.

## DISCUSSION

Brief inspection of table 2 is sufficient to show that the quality of the animal protein concentrate has a profound influence on chick growth. For example, the minimum average weight at 6 weeks in trial II was 119 gm. while the maximum was 321. In trial III, the corresponding weights were 89 and 379 gm., respectively. Since preliminary experiments indicated that 2 per cent of a dried brewer's yeast was adequate as a supplementary source of vitamin G in these rations, it is very unlikely that the growth results were influenced by a deficiency of this vitamin, especially where 3 per cent of the yeast was used. The variation in growth can fairly be attributed to the variation in nutritive value of the supplementary protein concentrates.

To some extent the nutritive values of animal protein concentrates have been investigated from the standpoint of type and condition of raw materials, methods of cooking and methods of drying and some explanations have been advanced as to the causes of variation in nutritive values.

In regard to type of raw materials, Hoagland and Snider ('26, '26 a, '27) have shown wide differences in the nutritive value of different animal organs and tissues. Mitchell and Carman ('26) and Mitchell, Beadles and Kruger ('27) demonstrated that meats containing higher amounts of connective tissue were inferior in nutritive value. Similar results have been obtained by Pittman, McCammon and Holman ('34). Meal prepared from the edible portions of fish was superior to and more digestible than meal prepared from cannery fish waste, in the experiments of Wilder, Bethke and Record ('34).

Curtis, Hauge and Kraybill ('32) showed that the hot-water-soluble fraction of tankage and similar products was inadequate, even for maintenance, while the hot-water-insoluble fraction was slightly superior to the original materials. A

similar experiment with haddock meal by Wilder, Bethke and Record ('34) gave a biological value of 34 for the hot-water-soluble fraction as compared to 97 for the insoluble fraction. Both investigations indicated that when the 'stick' or water extract was included in the final product the nutritive value was lowered.

The only protein recognizable as such in the hot-water-soluble fraction of a well-cooked animal protein concentrate is gelatin derived from the collagen of cartilage, connective tissues, skin and bone. The nutritional inadequacy of gelatin has been demonstrated by Jackson, Sommer and Rose ('28) and by Jones and Nelson ('31). These workers were unable to improve the nutritive value of gelatin by supplying the amino acids in which gelatin is known to be deficient.

Ingvaldsen ('29 a) has shown that putrefaction of the raw materials has a destructive effect on essential amino acids in fish meals and greatly increases the proportion of water-soluble nitrogen. He also found ('29) that high temperatures caused a diminution of the arginine and cystine in fish meals.

Morgan and Kern ('34) concluded that cooking caused a heat injury to meat protein which increased in severity with the length of exposure and the height of the temperature reached. Morgan ('31) and Fixsen and Jackson ('32) agree that the dry heating of casein causes a marked decline in its nutritive value. Evidence was found by Record, Bethke, Wilder and Kennard ('34) and by Record, Bethke and Wilder ('34) that higher drying temperatures adversely affected the nutritive values of fish meals, causing lowered digestibility and lowered biological value of the digestible portion.

Maynard, Bender and McKay ('32) found vacuum-dried white fish meal (cod and haddock) superior to flame-dried menhaden meal in growth promoting qualities. Maynard and Tunnison ('32) concluded that the superior nutritive value of the white fish meal was caused by the lower drying temperature and by the better quality of the raw material. Schneider ('32), who studied the same samples, showed that this superiority was due both to a higher digestibility and to a better utilization of the digestible fraction.

It is an obvious fact that the presence of undigestible protein reduces the nutritive value of a protein concentrate. According to Armsby and Moulton ('25), researches by Stuttzer in 1881, Pfeiffer in 1886 and by Kühn in 1894 showed that in vitro tests for undigestible protein gave results which agreed closely with biological tests. In other words, artificial and natural digestion of protein yielded comparable results when the latter was computed on the basis of the pepsin-insoluble nitrogen of the feces. Morgan and his associates ('09) obtained results which seemed to confirm this conclusion fully. It is reasonable to assume, therefore, that the portion of the animal protein concentrate not dissolved by prolonged treatment with pepsin at the optimum conditions for this enzyme is of negligible nutritive value.

The above literature has been cited in some detail at this point because it served as a basis for further efforts to explain the widely different nutritive values of the products used in the present tests. Although the nature and method of manufacture of most of these products was well known, there seemed little purpose in relying on manufacturers data since the product of a given plant may vary considerably. Efforts were directed toward the chemical investigation of the samples, making use of suggestions found in the work cited in an effort to detect criteria of quality which would apply in a general way to all the meat industry by-products with which this report is primarily concerned.

After preliminary trials a scheme of chemical analysis was decided upon, which included the determination of the nitrogen[1] precipitated by cupric ion, the nitrogen not precipitated by cupric ion but precipitated by phosphotungstic acid, the nitrogen not dissolved after prolonged digestion with pepsin, and the nitrogen soluble in hot water but precipitated by cupric ion. The scheme was purposely kept simple and limited to the determination of those forms of nitrogen which were considered of the greatest influence on nutritive value.

[1] The term nitrogen is here employed for simplicity to indicate the nitrogen of various groups of substances.

The results obtained by the fractionation of the nitrogen according to this scheme are given in table 1. The analytical details are given below.

*Methods used in the fractionation of the nitrogen in animal protein concentrates*

| *Reagents* | |
|---|---|
| Copper sulphate solution | 100 gm. $CuSo_4 .5H_2O$ per liter |
| Alum solution | 100 gm. $NaAl(SO_4)_2 .12 H_2O$ per liter |
| Magnesium oxide | 50 gm. MgO suspended in 1 liter |
| Hydrochloric acid | 250 gm. HCl in 1 liter |
| Pepsin solution | 5 gm. pepsin in 1 liter |
| Phosphotungstic acid | 100 gm. $P_2O_5 .24 WO_3 .x H_2O$ in 1 liter |
| Acetate buffer | Approximately 1 molal, pH approximately 5 |

*Procedure.* A quantity of the concentrate containing 0.5 to 1.0 gm. of crude protein was placed in a porous alundum crucible and extracted with ethyl ether for approximately 24 hours in a Bailey-Walker apparatus. It was then transferred to a 250 cc. beaker, mixed with 80 cc. of water and heated on a steam bath for 30 minutes. Five cubic centimeters of alum reagent, 25 cc. of copper sulphate solution and 50 cc. of magnesium oxide suspension were then added. Heating on a steam bath was continued for 20 minutes longer. When cool, the precipitate was filtered off and washed three times with 25 cc. of 2 per cent copper sulphate solution. The nitrogen in the precipitate was determined.

The filtrate and washings from the above were boiled for 30 minutes with a slight excess of magnesium oxide to drive off ammonia and amines, filtered, washed and made acid with hydrochloric acid to about 1 N. Ten cubic centimeters of phosphotungstic acid were added and the solution was kept in an ice box over night. More phosphotungstic acid was added if precipitation had not been complete, but a large excess was avoided. The precipitate was filtered off, using a filter aid if necessary, washed once with ice cold 1 normal hydrochloric acid containing about 1 per cent of phosphotungstic acid, and the nitrogen content of the precipitate determined.

TABLE 1

*Analyses of animal protein concentrates*

| SAMPLE NO. | DESCRIPTION | PERCENTAGE OF TOTAL NITROGEN | | | | | PERCENTAGE CRUDE PROTEIN (N × 6.25) | PERCENTAGE TRYPTOPHANE IN CRUDE PROTEIN | PERCENTAGE CYSTINE IN CRUDE PROTEIN | PROTEIN QUALITY INDEX |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Copper precipitable | Phosphotungstic acid precipitable[1] | Undigestible | Hot water soluble protein | Non-protein | | | | |
| 42 | Casein | 95.2 | 0.9 | 7.5 | 0.8 | 3.9 | 79.0 | 1.50 | 0.20 | 87.6 |
| 44 | Sardine meal, steam dried | 97.6 | 0.3 | 10.9 | 2.8 | 2.1 | 67.2 | 2.01 | 0.45 | 85.1 |
| 48 | Dogfish meal | 67.3 | 13.8 | 21.4 | 17.0 | 18.9 | 72.3 | 0.78 | 0.86 | 41.2 |
| 63 | Tankage | 64.6 | 26.3 | 8.6 | 35.2 | 9.1 | 52.5 | .... | .... | 45.4 |
| 64 | Meat scrap, dry rendered | 90.4 | 4.1 | 9.5 | 14.9 | 5.5 | 56.8 | 1.12 | 0.49 | 73.6 |
| 65 | Cracklings | 86.2 | 4.2 | 9.3 | 13.9 | 9.6 | 49.7 | .... | .... | 70.2 |
| 66 | Cracklings | 90.7 | 6.0 | 12.8 | 19.6 | 3.3 | 53.2 | 1.06 | 0.89 | 68.5 |
| 69 | Tankage | 52.2 | 36.4 | 10.3 | 16.6 | 11.4 | 48.3 | 0.77 | 0.54 | 46.5 |
| 70 | Meat scrap, wet rendered | 85.0 | 11.9 | 11.6 | 15.9 | 3.1 | 56.2 | 0.62 | 0.71 | 68.6 |
| 71 | Tankage | 63.8 | 28.8 | 12.1 | 23.1 | 7.4 | 59.4 | 0.83 | 0.54 | 49.3 |
| 72 | Tankage | 71.0 | 21.0 | 18.9 | 17.7 | 8.0 | 52.4 | 0.96 | 0.76 | 49.9 |
| 73 | Beef, vacuum dried | 90.8 | 0.9 | 2.6 | 8.5 | 8.3 | 90.8 | 0.72 | 1.10 | 83.4 |
| 74 | Sardine meal, vacuum dried | 97.1 | 1.2 | 10.2 | 7.2 | 1.7 | 68.4 | .... | .... | 83.1 |
| 76 | Whale meat meal | 86.2 | 7.6 | 2.9 | 2.8 | 6.2 | 75.4 | 1.79 | 0.86 | 84.4 |
| 78 | Cracklings | 92.8 | 4.2 | 6.3 | 13.4 | 3.0 | 50.2 | 0.78 | 0.74 | 80.2 |
| 79 | Menhaden meal, steam dried | 93.0 | 0.8 | 8.3 | 4.0 | 6.2 | 63.0 | 1.87 | 0.96 | 82.6 |
| 81 | Meat scrap, dry rendered | 80.8 | 15.1 | 12.0 | 17.5 | 4.1 | 58.2 | 1.30 | 0.82 | 64.3 |
| 82 | Sardine meal, steam dried | 99.3 | 0.8 | 9.4 | 2.9 | 0.0 | 69.0 | 2.12 | 0.45 | 88.5 |
| 84 | Gelatin | 100.0 | 0.0 | 0.0 | 100.0 | 0.0 | 98.7 | .... | .... | 40.0 |
| 85 | Mixture of 82 and 78 | | | | | | | .... | .... | 84.3 |

[1] Obtained from the filtrate from the copper precipitation.

The undigestible protein was determined on a fresh 2 gm. sample which was extracted with ethyl ether as before and transferred to a 500 cc. shaking bottle. Two hundred and fifty cubic centimeters of water, 5 cc. of hydrochloric acid and 25 cc. of freshly prepared pepsin solution were added. The mixture was shaken for 25 hours at a temperature of 37°C. Then 5 cc. of hydrochloric acid were added and the mixture was allowed to stand for 24 hours more at a temperature of 37°C. The insoluble material was filtered and washed two or three times with hot water. To obtain clear filtrates a filter aid was occasionally necessary. The nitrogen in the undissolved residue was determined.

The hot-water-soluble protein was determined by extracting a 2 gm. sample with ethyl ether until most of the fat was removed, then boiling with 200 cc. of water containing 5 cc. of the acetate buffer for 30 minutes. The mixture was filtered while hot and washed several times with hot water. The filtrate and washings were treated with cupric ion and magnesium oxide as already described, and the nitrogen content of the copper precipitate determined.

*The copper precipitable nitrogen.* Cupric ion in the presence of dilute hydroxide ion is one of the most specific reagents for intact protein. The fraction of the total protein which is precipitated by copper decreases regularly as the degree of decomposition of the protein increases. However, peptones, peptides and amino acids present among the protein degradation products are also precipitated to some extent by copper. In the laboratory, steam, acid or enzymic hydrolysis of protein concentrates was found to cause a marked lowering of the copper-precipitable nitrogen.

The percentage of the total nitrogen which was precipitable by copper varied from 52.2 to 100 in the protein concentrates. It was particularly low in those concentrates, such as tankages, prepared by wet rendering processes and other processes allowing extensive decomposition.

*The phosphotungstic acid precipitable nitrogen.* The nitrogen not precipitated by copper cannot be regarded in all cases

as having no nutritive value. In order to estimate the portion of this nitrogen to which a nutritive value may be ascribed, phosphotungstic acid was employed as a precipitant for the peptones, peptides, and amino acids which escaped the copper treatment. These phosphotungstic acid precipitates were investigated by means of the formaldehyde titration. It was found that they gave comparatively large formaldehyde titration values which were increased five- to twelvefold by acid hydrolysis indicating an average composition corresponding to that of a complex peptide. After acid hydrolysis, 45 to 65 per cent of the nitrogen in these fractions was present in the form of alpha-amino nitrogen. It is evident, therefore, that such material must have a certain nutritive value since it contains a high proportion of amino acids, especially basic amino acids which are essential to nutrition. It may be seen from table 1 that as the percentage of copper-precipitable nitrogen decreased, the percentage of nitrogen precipitated by phosphotungstic acid tended to increase. This fraction, consisting mostly of peptones, peptides and amino acids resulting from protein disintegration, varied from 0.3 to 36.4 per cent. The material not precipitated by copper or by phosphotungstic acid rarely showed more than a slight increase in the formaldehyde titration after acid hydrolysis. Hence, it may be assumed that this non-precipitable nitrogen has a negligible nutritive value, although certain amino acids may be present in small quantities.

*The undigestible nitrogen.* Nitrogen in the form of undigestible protein or other insoluble nitrogenous residues was estimated by the failure of such nitrogen to dissolve when treated with pepsin and hydrochloric acid under the optimum conditions for this enzyme. This nitrogen is automatically included in the copper precipitable nitrogen and must be separately estimated since it may be present in considerable amounts.

A further treatment of the pepsin-insoluble residues with trypsin gave no appreciably greater degree of solution. Kühn in 1894 (cited by Armsby and Moulton, '25) found that

the prolonged treatment with pepsin was equivalent to the treatment with pepsin followed by trypsin. It was apparent that the pepsin treatment alone gave the minimum value for undigestible nitrogen, hence the supplementary action of trypsin was not employed. Materials which contribute to the undigestible fraction are the keratins of hoof, horn and hair, and protein material which has been extensively denatured by high temperatures. Values for undigestible nitrogen obtained from the animal protein concentrates varied from 0.0 to 21.4 per cent of the total nitrogen.

*The hot-water-soluble protein nitrogen.* The chief hot-water-soluble protein present in animal protein concentrates is gelatin. Its low nutritive value makes necessary its separate estimation apart from other proteins in the copper precipitable fraction. In materials made largely from bone, cartilage, connective tissue and skin, the gelatin content may be very high. While such products may show a high proportion of copper-precipitable nitrogen and a low proportion of undigestible nitrogen, their nutritive values remain relatively low.

Nitrogen in this form varied, in the commercial concentrates, from 0.8 to 35.2 per cent of the total nitrogen and reached a value of 100 in the case of the gelatin sample.

*The cystine and tryptophane content.* Analyses for cystine and tryptophane were made, using the methods of Ingvaldsen ('29), Baernstein ('30) and Folin and Ciocalteu ('27), in order to determine what relation to nutritive value such analyses may bear when applied to commercial concentrates. Since cystine and tryptophane are among the most labile of the essential amino acids, they are very likely to be partially or completely destroyed by putrefaction or adverse manufacturing conditions.

The percentage of tryptophane in the crude protein varied from 0.62 to 2.12. Cystine varied from 0.20 to 1.10 per cent of the crude protein. Both are practically absent in gelatin. No attempt was made to determine what proportions of these amino acids were in the undigestible fraction. Keratins, the

undigestible proteins of hoof, horn and hair, are especially rich in cystine, and are among those substances likely to be present in the undigestible fraction.

*Analytical characteristics of various classes of concentrates.* As a class, the tankages (samples 63, 69, 71 and 72) showed the lowest copper-precipitable fraction, the highest phosphotungstic-acid-precipitable fraction, the highest undigestible fraction, and the highest hot-water-soluble fraction. The dogfish meal (sample 48) may also be classed with these from the analytical standpoint.

The meat scraps (samples 64, 70 and 81) showed little marked difference as a class from the cracklings (samples 65, 66 and 78). These groups appeared definitely superior to the tankages in regard to the above analytical characteristics.

The vacuum-dried beef (sample 73) and the whale meat meal (sample 76) gave the most favorable analyses of the meat products. The fish meals included in the test gave results which were equally good, with the exception of the dogfish meal. However, the investigation at the present stage has not included a sufficient number of samples of fish meals to warrant final comparisons in this report.

It may be pointed out that the method of analysis employed provides strong evidence of the condition and type of raw materials and of the effect of the manufacturing process on the quality of the product. Decomposition caused by putrefaction, autolysis or poor methods of manufacture is reflected in a decrease of the copper-precipitable-fraction and an increase in the phosphotungstic-acid-precipitable fraction. The inclusion of undigestible nitrogenous substances and the effect of excessive temperatures introduce an unduly large undigestible fraction. An increase in the cartilage, connective tissue, skin and bone content causes a corresponding increase in the hot-water-soluble protein nitrogen. The same is true when the 'stick' is included in the final product. Finally, the presence of much nitrogen in the form of ammonia, amines, urea and similar types of non-protein nitrogen causes a wide discrepancy to appear between the summation of the nitrogen

accounted for by the copper and phosphotungstic acid treatments and the total nitrogen.

It should also be pointed out that the analytical scheme which was employed does not detect the presence of toxic substances. Mortality, the only criterion of toxicity which was available in these experiments, was very low during the last 2 weeks of the trials and actually lower in the slow growing groups than in the faster growing groups, hence it would appear that toxicity was not an important factor in the growth results herein reported.

*Relation of analytical data to growth data*

In comparing the average weights of chicks at 6 weeks with the percentages of total nitrogen precipitated by copper, it was observed that this analytical characteristic showed a moderately good correlation with nutritive value. Samples of low nutritive value invariably had a low percentage of copper-precipitable nitrogen, while samples of high value had a high percentage. However, a high percentage of copper-precipitable nitrogen does not assure a high nutritive value, because of the possible inclusion of undigestible nitrogen and of the nitrogen of gelatin. On the other hand, a low percentage of copper-precipitable nitrogen is an excellent criterion of low nutritive value.

Proceeding a step farther, it was noted that subtraction of the undigestible nitrogen from the copper-precipitable nitrogen gave a numerical expression of quality which showed a distinctly better correlation with growth, especially among the concentrates of lower quality. This result suggested that, if the phosphotungstic-acid-precipitable nitrogen and the hot-water-soluble protein nitrogen could also be combined into the numerical expression of protein quality, the agreement of the analytical and biological results might be further improved. Since both of these fractions must be assumed to possess a lower nutritive value than corresponding quantities of undecomposed meat protein, the chief problem appeared to be the choice of a fractional factor which would express their low

value. By a process of trial it was found that, when both of these low value fractions were assigned a factor of four-tenths, the best agreement of analytical and biological results was obtained. We have on this basis, calculated for each protein concentrate an expression called the protein quality index which is based on the analytical results given in table 1. The formula for this index is:

Protein quality index = A — (B + .6 C) + .4 D
where A = Percentage of total nitrogen precipitated by copper (inclusive of B and C).
B = Percentage of total nitrogen not digestible.
C = Percentage of total nitrogen as hot-water-soluble protein.
D = Percentage of total nitrogen precipitated by phosphotungstic acid.

The values of this index for each protein concentrate are given in table 1. These indices varied from 40.0 to 88.5. On this basis, none of the concentrates represented a perfect animal protein, as such a protein would have an index of 100.

In table 2 it may be seen that the protein quality index shows a close agreement with both the total growth and the gain per unit of feed consumed during the last 2 weeks of the period. Perhaps the best way in which to express this agreement between sets of data obtained by such widely different methods is the graphical method. Figures 1 and 2 present a graphical comparison of total growth and growth per unit of feed consumed with the protein quality indices of the concentrates.

Within the limits of the expected variation, samples retained their same relative positions whether plotted on the basis of total growth or on the basis of growth per unit of feed consumed. Exceptions to this rule seemed to occur in the cases of samples 70, 78 and 85 which lagged in total growth produced but fell closely into line in respect to growth produced per unit of feed consumed during the last 2 weeks. It is possible that such results might be caused by unpalatability or any other factor causing lowered feed consumption.

Sample 85, which was composed of equal parts of protein from samples 78 and 82, gave results for total growth and growth per unit of feed consumed which were intermediate

between those obtained from 78 and 82 (cracklings and sardine meal, respectively). This result is in agreement with the report of Record, Bethke, Wilder and Kennard ('34).

TABLE 2

*Growth data of trials I, II and III*

| SAMPLE NO. | PROTEIN QUALITY INDEX | NUMBER OF CHICKS AT 6 WEEKS | AVERAGE WEIGHT OF CHICKS AT 6 WEEKS IN GRAMS | AVERAGE GAIN PER UNIT OF FEED, LAST 2 WEEKS |
|---|---|---|---|---|
| **Trial I** | | | | |
| 42 | 87.6 | 13 | 257.0 | |
| 63 | 45.4 | 13 | 172.0 | |
| 64 | 73.6 | 13 | 240.7 | |
| 65 | 70.2 | 12 | 230.0 | |
| 66 | 68.5 | 8 | 232.1 | |
| 69 | 46.5 | 11 | 151.9 | |
| 70 | 68.6 | 12 | 175.2 | |
| 71 | 49.3 | 12 | 162.0 | |
| 72 | 49.9 | 12 | 135.5 | |
| **Trial II** | | | | |
| 42 | 87.6 | 15 | 269.0 | 0.351 |
| 44 | 85.1 | 12 | 321.0 | 0.346 |
| 48 | 41.2 | 13 | 119.0 | 0.157 |
| 64 | 73.6 | 14 | 262.8 | ....[1] |
| 66 | 68.5 | 13 | 229.5 | 0.269 |
| 69 | 46.5 | 13 | 144.7 | 0.181 |
| 70 | 68.6 | 11 | 200.2 | 0.292 |
| 71 | 49.3 | 15 | 169.5 | 0.235 |
| 72 | 49.9 | 13 | 141.2 | 0.150 |
| 73 | 83.4 | 14 | 278.5 | 0.349 |
| **Trial III** | | | | |
| 70 | 68.6 | 14 | 231.5 | 0.322 |
| 71 | 49.3 | 21 | 167.3 | 0.244 |
| 72 | 49.9 | 14 | 168.5 | 0.262 |
| 76 | 84.4 | 10 | 377.0 | 0.370 |
| 78 | 80.2 | 28 | 230.8 | 0.337 |
| 79 | 82.6 | 13 | 363.0 | 0.389 |
| 81 | 64.3 | 15 | 255.9 | 0.336 |
| 82 | 88.5 | 23 | 379.0 | 0.369 |
| 84 | 40.0 | 11 | 89.0 | ....[1] |
| 85 | 84.3 | 20 | 289.8 | 0.357 |

[1] Unknown quantity of feed lost.

It is evident that chemical analyses such as have been described are capable of throwing much light on the variation of protein quality, one of the major problems in animal nutrition. Further investigation and application of chemical methods may lead to greater refinement and improved signifi-

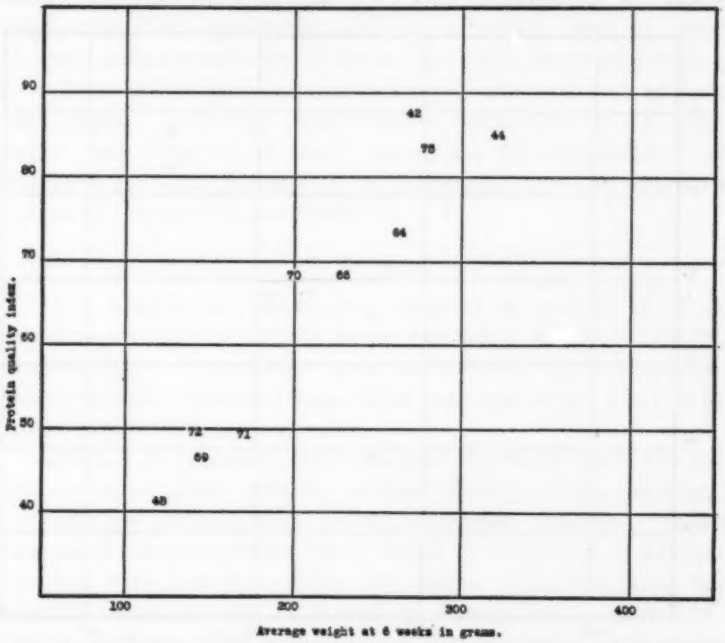


Fig. 1 Total average weight at 6 weeks obtained in trial I, plotted against protein quality index. Plotted numbers refer to animal protein samples as in table 1.

cance of results. We have hesitated, however, to complicate the procedure beyond the bounds of practical applicability, preferring to ascertain first whether the simplest technic will provide a sufficiently accurate result for the purpose. It is believed that the procedure employed is the simplest which conforms to theoretical requirements.

It is very difficult to interpret amino acid levels in the complex animal and vegetable protein mixtures required by chicks. Partial deficiencies of amino acids may, to a large extent, be rectified by feeding higher levels of protein or by feeding supplementary sources of these amino acids. Moreover, the digestibility and the state of disintegration of the

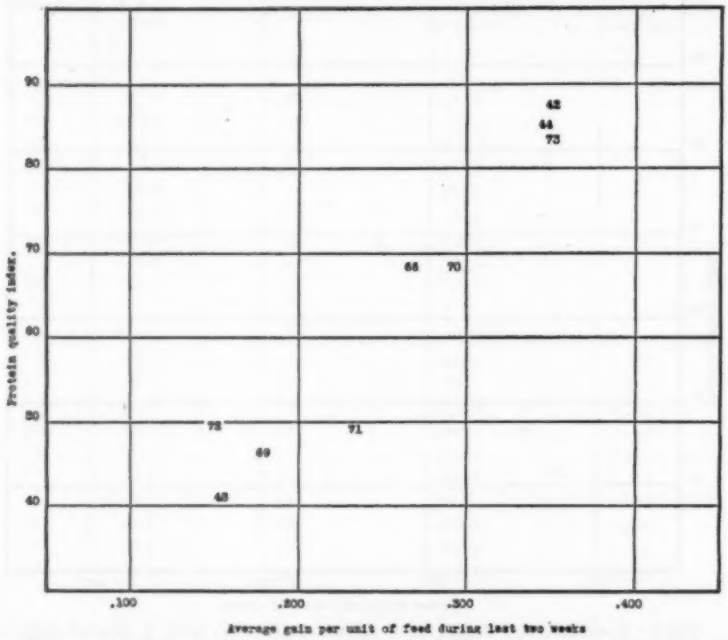


Fig. 2 Total average weight at 6 weeks obtained in trial II plotted against protein quality index.

protein may exert a large effect on its nutritive value apart from its amino acid composition.

The cystine and tryptophane content of the animal protein concentrates could not be used to predict their nutritive values. Sample 48 which gave very poor growth contained more cystine and tryptophane than did several other samples which greatly exceeded it in nutritive value. Sample 81 was

well supplied with both cystine and tryptophane and yet gave inferior results. The fact that casein is especially low in cystine seems to have had no influence on its excellent growth-promoting action.

As a further index to protein decomposition, the samples were tested for the presence of hydrogen sulphide by mixing them with dilute sulphuric acid and allowing them to stand at room temperature for 24 hours in a flask stoppered with a cork from which was suspended a moist strip of lead acetate test paper. Although considerable darkening of the test paper was obtained in many instances, no correlation was observed between the degree of darkening and the nutritive values of the protein concentrates.

## SUMMARY

When used as supplementary sources of protein in chick growing rations, tankages gave decidedly inferior results, meat scraps and cracklings gave much better but yet unsatisfactory results, while vacuum-dried beef and whale meat meal gave very satisfactory results.

Analyses of animal protein concentrates were made for the estimation of intact protein, protein decomposition products, undigestible protein and hot-water-soluble protein. These analytical characteristics were found to exhibit a good correlation with nutritive value for chicks, when assigned the relative values of 100, 40, 0 and 40, respectively. Such analytical methods offer a possibility for the rapid laboratory determination of protein quality in commercial concentrates.

The cystine, tryptophane and hydrogen sulphide content of the protein concentrates could not be used as a basis for predicting their nutritive values.

## LITERATURE CITED

ALLARDYCE, W. J., W. HENDERSON AND V. S. ASMUNDSON 1933 Fish meal supplements for chicks. III. Poul. Sci., vol. 12, p. 163.

ARMSBY, H. P., AND C. R. MOULTON 1925 The animal as a converter of matter and energy. Am. Chem. Soc. Mon. Series, No 23. Chem. Cat. Co., New York, N. Y., pp. 86–87.

BAERNSTEIN, H. D. 1930 The gasometric determination of cysteine and cystine. J. Biol. Chem., vol. 89, p. 125.

CURTIS, P. B., S. M. HAUGE AND H. R. KRAYBILL 1932 The nutritive value of certain animal protein concentrates. J. Nutrition, vol. 5, p. 503.

FIXSEN, M. A. B., AND H. M. JACKSON 1932 The biological value of proteins. IV. Biochem. J., vol. 26, p. 1923.

FOLIN, O., AND V. CIOCALTEU 1927 On tyrosine and tryptophane determinations in proteins. J. Biol. Chem., vol. 73, p. 627.

HOAGLAND, R., AND G. C. SNIDER 1926 Nutritive value of the proteins in veal and calf sweetbreads; in beef cheek meat, lips, tongues, brains, spleen and tripe; and in hog brains and tongues. J. Agr. Res., vol. 32, p. 679.

———— 1926 a Nutritive value of the protein in voluntary muscle, heart, liver and kidney from cattle, sheep and hogs. J. Agr. Res., vol. 32, p. 1025.

———— 1927 The value of beef protein as a supplement to the proteins in certain vegetable products. J. Agr. Res., vol. 34, p. 297.

INGVALDSEN, T. 1929 Fish meals. I. The effect of high temperature employed for drying on the nitrogen partition in fish meals. Can. Chem. Met., vol. 13, p. 97.

———— 1929 a Fish meals. II. Comparative analysis of meals made from non-putrid and putrid materials. Can. Chem. Met., vol. 13, p. 129.

JACKSON, R. W., B. E. SOMMER AND W. C. ROSE 1928 Experiments on the nutritive properties of gelatin. J. Biol. Chem., vol. 80, p. 167.

JOHNSON, O., AND D. BRAZIE 1934 Comparative value of some commercial protein supplements in the ration of growing chicks. J. Agr. Res., vol. 48, p. 183.

JONES, D. B., AND E. M. NELSON 1931 Nutritive value of potato protein and of gelatin. J. Biol. Chem., vol. 91, p. 705.

MAYNARD, L. A., R. C. BENDER AND C. M. MCKAY 1932 Vitamin A and protein content of various fish meals. J. Agr. Res., vol. 44, p. 591.

MAYNARD, L. A., AND A. V. TUNISON 1932 Influence of drying temperatures upon digestibility and biological value of fish proteins. Ind. Eng. Chem., vol. 24, p. 1168.

MITCHELL, H. H., J. R. BEADLES AND J. H. KRUGER 1927 The relation of the connective tissue content of meat to the protein value in nutrition. J. Biol. Chem., vol. 73, p. 767.

MITCHELL, H. H., AND G. G. CARMAN 1926 The biological value of the nitrogen of mixtures of patent white flour and animal foods. J. Biol. Chem., vol. 68, p. 183.

MORGAN, A. F. 1931 The effect of heat upon the biological value of cereal proteins and casein. J. Biol. Chem., vol. 90, p. 771.

MORGAN, A. F., AND G. E. KERN 1934 The effect of heat upon the biological value of meat protein. J. Nutrition, vol. 7, p. 367.

MORGAN, A., C. BEGER AND F. WESTHAUSSER 1909 Weitere Untersuchungen über die Verwertung der nicht-eiweissartigen Stieksoffverbindungen der Futtermittel sowie der Ammonsalze durch das milchgebende Tier. Landw. Vers. Stat., vol. 71, p. 1.

PRANGE, R. W., C. W. CARRICK AND S. M. HAUGE 1928 Growth value of proteins from commercial animal products. I. Poul. Sci., vol. 7, p. 95.

———— 1928 a Growth value of proteins from commercial animal products. II. Poul. Sci., vol. 7, p. 186.

———— 1928 b Growth value of proteins from commercial animal products. III. Poul. Sci., vol. 7, p. 233.

PITTMAN, M. S., R. B MCCAMMON AND M. HOLMAN 1934 Utilization of meat by human subjects. I. J. Nutrition, vol. 8, p. 503.

RECORD, P. R., R. M. BETHKE AND O. H. M WILDER 1934 Effect of method of manufacture on the nutritive value of fish meals as determined by growth studies with chicks. J. Agr. Res., vol. 49, p. 715.

RECORD, P. R., R. M. BETHKE, O. H. M. WILDER AND D. C. KENNARD 1934 The comparative nutritive value of different fish meals for chicks. Poul. Sci., vol. 13, p. 259.

SCHNEIDER, B. H. 1932 Nitrogen balance studies with various fish meals. J. Agr. Res., vol. 44, p. 723.

TITUS, H. W., E. MCNALLY AND F. C. HILBERG 1930 A comparison of shrimp bran and two kinds of fish meal. Poul. Sci., vol. 9, p. 219.

WILDER, O. H. M., R. M. BETHKE AND P. R. RECORD 1934 Effect of method of manufacture on the nutritive value of fish meals as determined by nitrogen balance studies with rats. J. Agr. Res., vol. 49, p. 723.

# A NEW TOXICANT OCCURRING NATURALLY IN CERTAIN SAMPLES OF PLANT FOODSTUFFS[1]

## IX. TOXIC EFFECTS OF ORALLY INGESTED SELENIUM

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FIVE FIGURES

(Received for publication March 8, 1935)

The presence of selenium (Robinson, '33) in the toxic grain used in the experiments described by Franke ('34 b) made it desirable to investigate the physiological effects of selenium compounds. The occurrence of selenium in foodstuffs gives a new aspect to the toxicology of the element. In the past the physiological effects of selenium have been studied by injection methods in the majority of cases. The authors are not aware of any published work which has involved the ad lib. feeding of artificial selenium-containing diets.

The work reported in this paper was planned in order to compare the effects produced by artificial selenium-containing diets and the effects produced by the affected natural foodstuffs which have previously been described by the authors. At present considerable attention is being focused on 'the selenium problem,' and there may be a tendency to regard selenium as the sole causative factor in the production of the natural plant toxicants. Although the present paper will

[1] Published with the permission of the director of the South Dakota Agricultural Experiment Station as communication no. 12 from the Department of Experiment Station Chemistry. These investigations are being carried out under the Purnell Fund and with the cooperation of the Bureau of Chemistry and Soils, Bureau of Plant Industry, Bureau of Animal Industry, and Bureau of Home Economics of the United States Department of Agriculture.

point out a number of similar physiological effects produced by the natural toxicant and the element selenium, it has not definitely been proved that the toxicity of cereal grains is strictly proportional to their selenium content.

The toxicity of selenium depends upon the ionic combination in which it is found. Thus, elemental selenium, in the form of the powdered metal, seems to be virtually harmless when added to control wheat in concentrations comparable to the amount present in toxic wheat. At the same level, sodium selenide produces marked growth depression, but no deaths occur. When fed in the form of sodium selenite or selenate, selenium produces toxic effects approaching those of the natural toxicants. Sodium selenite has probably been investigated more frequently than any other form of selenium (Jones, '09; Cathcart and Orr, '14; Levine and Flaherty, '26).

## EXPERIMENTAL

In the present experiment, various amounts of sodium selenite were added to control wheat diets which were then fed ad lib. to white rats to determine the effect on the growth, food intake, hemoglobin, and gross pathology. Stained smears of the blood were also examined at frequent intervals.

The rats used were of Wistar Institute origin. They had been weaned at 21 days and maintained for 1 week on McCollum's diet I as described by Burr and Burr ('29). They were then divided into four groups of nine rats each, and placed in the individual drawer cages described by Franke and Franke ('34), and given diets as follows:

Group 1 received control wheat diet. Groups 2, 3, and 4 received the control wheat diet plus 22.3, 33.5, and 52.1 parts per million, respectively, of selenium in the form of sodium selenite.

The composition of the diet used is described in a former publication (Franke and Potter, '34). Selenium was added by pipetting the proper amount of a solution of sodium selenite[3] (2.976 mg. Se per cubic centimeter) directly into a

[3] C. P. obtained from Eimer and Amend.

pan containing 1 kilo of diet which was then dried, at room temperature, and thoroughly mixed.

Fresh distilled water containing a trace of iodine was available to the rats at all times.

The rats were weighed at 5-day intervals, and their food intake was recorded daily.

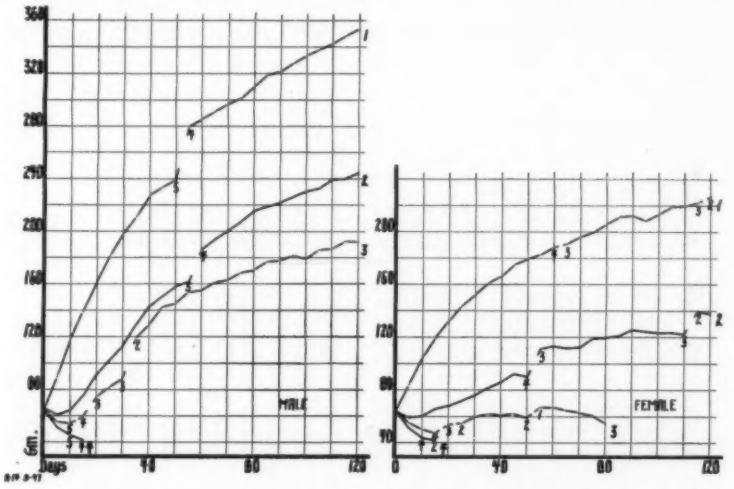


Fig. 1 Growth curves for males and females on following diets: 1) Control wheat; 2) control wheat + 22.3 parts per million of selenium as sodium selenite; 3) control wheat + 33.5 parts per million of selenium as sodium selenite; 4) control wheat + 52.1 parts per million of selenium as sodium selenite. Figures under curves indicate number of surviving animals.

Hemoglobin was determined, as in the previous paper, with greater intervals between bleedings as the experiment progressed. Blood smears were made from the same samples which were used for hemoglobin determinations and treated[3] with Wright's stain.[4]

[3] It was found convenient to add stain and buffer from two 10-cc. syringes, using 0.8 cc. of each solution per slide.

[4] Osgood and Haskins, Laboratory Diagnosis, 1931.

## DISCUSSION OF RESULTS

The experiment was terminated 359 days after it had begun. At this time there were four survivors on the 22.3 p.p.m. diet, and only one rat on the 33.5 p.p.m. diet. Three of the control animals were sacrificed during the experiment. The animals which received 52.1 p.p.m. had all died by the seventeenth day of the experiment. The growth of the rats in the various groups for the first 120 days is shown in figure 1. The growth

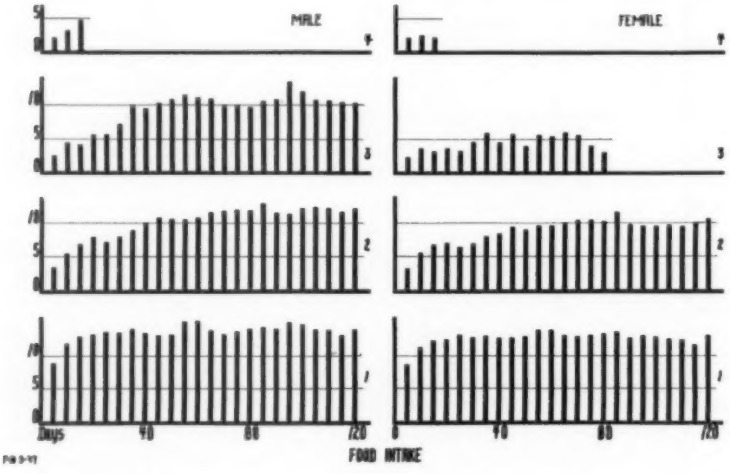


Fig. 2 Food intake per rat per day. Five-day averages. Groups numbered as in figure 1. Figures based on surviving animals as in figure 1.

is probably a direct result of the voluntary restriction of food intake, as was found to be the case with the natural toxicant (Franke and Potter, '34). The food consumption for the various groups is shown in figure 2. The food intake bears an inverse relation to the selenium content. In an experiment in which rats were given a choice between diets having various concentrations of selenium, it was found that the animals invariably chose the least toxic diet. They were apparently able to taste the selenium in the diets. However, it seems unlikely that a disagreeable taste could account for

the degree of inanition which occurred in group 4. In support of the latter idea is the fact that anorexia has been reported in nearly every instance where selenite has been injected. In a case in this laboratory, a rat fasted completely for 13 days following an injection of sodium selenite (3.7 mg. per kilo).

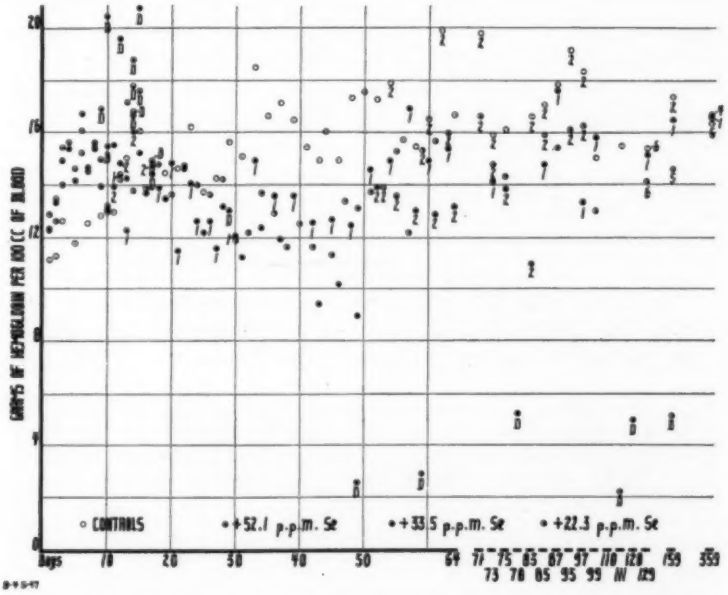


Fig. 3 Hemoglobin levels for the experimental period. Each point represents three rats unless otherwise indicated by sub-figures. The final hemoglobin levels reached by individual rats are indicated by D.

The hemoglobin levels (fig. 3) attained by the rats in the various groups show two distinct trends. Prior to a certain critical period (the ninth to seventeenth day of the experiment) the experimental rats show abnormally high levels of hemoglobin. After that date there is a distinct tendency toward anemia. High levels of hemoglobin are probably a characteristic symptom of acute selenium poisoning. The absolute amount of hemoglobin is almost certainly not increased since the blood is probably anhydremic. In this

laboratory, Painter (unpublished data) observed hemoglobin levels as high as 25 gm. per 100 cc. of blood in rats which

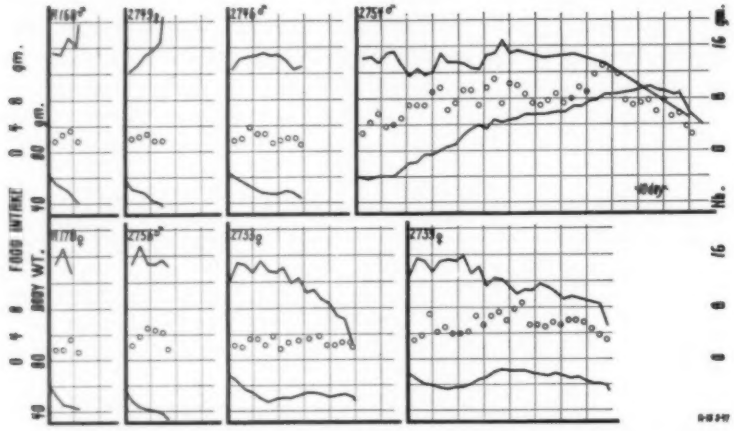


Fig. 4 Hemoglobin, growth, and food intake of individual rats in group 3. (33.5 p.p.m. Se as $Na_2SeO_3$).

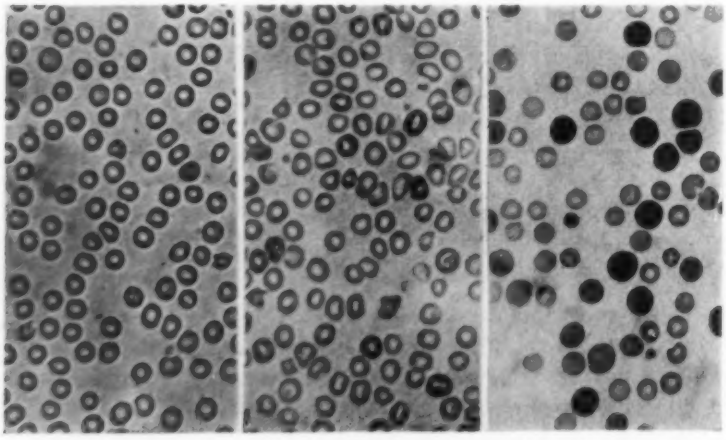

*Fig. A* Left: erythrocytes from a control rat (M 22); middle: erythrocytes with achromia (M 42); right: anisocytosis and polychromatophilic macrocytes (M 46). Magnified 500 diameters. E—22 orange color filter. Orthochromatic film.

were injected with lethal doses of sodium selenite. Post mortems on injected rats reveal conditions which are almost identical with those found in the rats in this series which died with high hemoglobin levels. The anhydremia can be explained in a majority of cases by the presence of considerable fluid in either the thoracic or the abdominal cavities, or in both.

Figure 3 shows that all (nine) of the rats on 52.1 p.p.m., four of the rats on 33.5 p.p.m., and none of the rats on 22.3 p.p.m. died during the so-called critical period. Most of these rats had extremely high levels of hemoglobin, and all of the rats showed acute symptoms at the post mortem.

Figure 4 shows the hemoglobin, growth and food intake for individual rats in group 3 (33.5 p.p.m.). This set of curves compares quite closely with those obtained by Franke and Potter ('34) in which the natural toxicant was used. It is easily seen, however, that the effects produced in this group were less severe than the effects produced in series 87 in the preceding paper. This is well illustrated by the fact that in the former series the food intake per rat per day was only 2.9 gm., while in this group it was 5.1 gm.

After the critical period a number of rats began to show decreases in hemoglobin and eventually developed a fatal anemia. Blood smears made throughout the course of the experiment frequently showed achromia, and later anisocytosis with polychromatophilic macrocytes (fig. A), in the anemic rats. Among the acute cases there was a slight anisocytosis with a tendency toward leucopenia. The latter symptom was observed by Jones ('12) in acute cases after injection.

Some of the rats seemed practically unaffected by the toxic diets, both from the standpoint of hemoglobin and body weight. These rats included one male on the 33.5 p.p.m. diet, and three males and two females on the 22.3 p.p.m. diet. These rats were tested for fertility by various cross matings. The only matings which proved fertile were those in which

either the male or the female was a control animal. Under these conditions, the males on 33.5 p.p.m. were able to reproduce, and one of the females cast a litter. The results seem to bear out the conclusions of Macomber ('23) in regard to the 'fertility threshold.' It is quite possible that the fertility of the experimental rats was lowered to such an extent that fertile matings could result only with control rats of high fertility. More work will need to be done on this point before definite conclusions can be reached.

The pathological symptoms readily classify into two types which are strictly analogous to those described by Franke ('34 a) as subacute and chronic. The subacute cases have practically the same post mortem appearance as rats which have been injected with a lethal dose of selenium, except that they are always emaciated. The surviving rats were killed on the three hundred and fifty-ninth day, and although their hemoglobin was normal, the livers had the characteristic hobnailed appearance which is caused by necrosis and regeneration. The case of one of the females on 33.5 p.p.m. was unique. This animal died in the latter part of the experiment with what might be considered acute symptoms. The liver showed typical lesions, but the animal was distinctly not anemic. The thorax was filled with a transudate, and the lungs were completely atelectatic. Terminal hemoglobin was not determined.

In the present investigation the sodium selenite used was assumed to be in the form of the anhydrous salt. By reducing the selenite to its elemental form, it was possible to recover 98.2 per cent of the theoretical amount of selenium. Marked differences in the solubility of samples of sodium selenite have been found, and there is some evidence that variations in toxicity may occur.

It is possible that a portion of the added selenite was reduced by contact with the diet. The authors have been unable completely to prove that no reduction occurred. However, it has been shown that elemental selenium is formed when sodium selenite is reduced and that at this level elemental

selenium is non-toxic. The toxicity of the diets containing selenite indicates per se that any reduction which occurred must have been slight.

## CONCLUSION

The symptoms of selenium poisoning, produced by feeding small quantities of sodium selenite, in an otherwise normal diet, are virtually identical with the symptoms produced by the natural plant toxicant described by the authors elsewhere (Franke, '34 a; Franke and Potter, '34). Symptoms considered are 1) growth, 2) food intake, 3) hemoglobin levels, 4) gross pathology. It is realized that these factors alone are far from conclusive. They do, however, support the idea that selenium is very closely connected with the natural toxicant.

## LITERATURE CITED


BURR, G. O., AND M. M. BURR 1929 A new deficiency disease produced by the rigid exclusion of fat from the diet. J. Biol. Chem., vol. 82, p. 345.

CATHCART, E. P., AND J. B. ORR 1914 The influence of carbohydrates and fat on protein metabolism. III. The effect of sodium selenite. J. Physiol., vol. 48, p. 113.

FRANKE, KURT W. 1934 a A new toxicant occurring naturally in certain samples of plant foodstuffs. I. Results obtained in preliminary feeding trials. J. Nutrition, vol. 8, p. 597.

———— 1934 b A new toxicant occurring naturally in certain samples of plant foodstuffs. II. The occurrence of the toxicant in the protein fraction. J. Nutrition, vol. 8, p. 609.

FRANKE, KURT W., AND VAN R. POTTER 1934 A new toxicant occurring naturally in certain samples of plant foodstuffs. III. Hemoglobin levels observed in white rats which were fed toxic wheat. J. Nutrition, vol. 8, p. 615.

FRANKE, KURT W., AND W. R. FRANKE 1934 A metabolism cage for rats. J. Lab. and Clin. Med., vol. 19, p. 669.

JONES, CHARLES O. 1909 The physiological effects of selenium compounds with relation to their action on glycogen and sugar derivatives in the tissues. Biochem. J., vol. 4, p. 405.

———— 1912 The action of selenium salts on red blood corpuscles. Biochem. J., vol. 6, p. 106.

LEVINE, V. E., AND R. A. FLAHERTY 1926 Hypoglycemia induced by sodium selenite. Proc. Soc. Exptl. Biol. Med., vol. 24, p. 251.

MACOMBER, DONALD 1923 The threshold of fertility in rats and its relation to dietary deficiency. Am. Naturalist, vol. 57, p. 519.

ROBINSON, W. O. 1933 Determination of selenium in wheat and soils. J. Assoc. Official Agr. Chem., vol. 16, p. 423.

# A NEW TOXICANT OCCURRING NATURALLY IN CERTAIN SAMPLES OF PLANT FOODSTUFFS[1]

## X. THE EFFECT OF FEEDING TOXIC FOODSTUFFS IN VARYING AMOUNTS, AND FOR DIFFERENT TIME PERIODS

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FOUR FIGURES

(Received for publication March 8, 1935)

Two series of feeding experiments were carried out on rats: the first series was fed varying amounts of toxic corn in the diet, and the second series was fed a diet containing toxic wheat. These series were intended to simulate: 1) the feeding of individual samples of varying degrees of toxicity occurring in naturally grown foodstuffs (Franke, '34), and 2) the effect on the growth of animals by changing the diet containing toxic grain to one containing control grain.

### *Feeding varying amounts of toxic corn in the diet*

The rats used in these two series were of Wistar Institute origin and had been weaned at 21 days of age and maintained for 1 week on a stock diet. Litter mates were then divided into the required number of groups of ten rats each so that each group contained the same number of males and females, and weighed approximately the same. The rats were weighed every 5 days and the daily food intake recorded.

[1] Published with the permission of the director of the South Dakota Agricultural Experiment Station as communication no. 14 from the Department of Experiment Station Chemistry. These investigations are being carried out under the Purnell Fund and with the cooperation of the Bureau of Chemistry and Soils, Bureau of Plant Industry, Bureau of Animal Industry, and Bureau of Home Economics of the United States Department of Agriculture.

The composition of the diet was as follows:

| | *Per cent* |
|---|---|
| Ground whole corn | 70.0 |
| Casein | 11.0 |
| Sucrose | 15.0 |
| Lard | 2.0 |
| Calcium carbonate | 1.4 |
| Sodium chloride | 0.6 |
| Total | 100.0 |

Each rat was kept in an individual cage as described by Burr and Burr ('29). The diets of the five groups of ten rats each varied in the corn content as follows:

| *Group* | *Per cent of corn fraction of diet* | | *Per cent of total diet* |
|---|---|---|---|
| A | Toxic corn lab. no. 388 | 0.0 | 0.0 |
| B | Toxic corn lab. no. 388 | 25.0 | 17.5 |
| C | Toxic corn lab no. 388 | 50.0 | 35.0 |
| D | Toxic corn lab. no. 388 | 75.0 | 52.5 |
| E | Toxic corn lab. no. 388 | 100.0 | 70.0 |

The average growth of these groups is given for the males in figure 1, and for the females in figure 2. It is of interest to note that group B, which received only 17.5 per cent of toxic corn lab. no. 388, had a decided lower growth than control group A, and that two out of ten rats had died by the one hundred and fiftieth day of experimentation. The growth of group C, receiving 35 per cent toxic corn, was still lower, and four of the ten animals died. Where the diet contained 52.5 per cent toxic corn (group D), all the rats had died by the one hundred and twenty-third day, and in group E, receiving 70 per cent toxic corn, all had died by the ninetieth day.

The external appearance of the rats in group B did not differ much from those in group A except for size, but in the other groups decided differences were noted. All gradations from normal to extremely abnormal animals, both as to growth and pathology, were noted as described before (Franke, l.c.).

As the per cent of toxic foodstuff in the diet increased, the food consumption decreased. The average food consumption for group A was 13.16 gm.; B, 10.46 gm.; C, 8.21 gm.; D, 5.92 gm.; and E, 4.74 gm. per rat day to the ninetieth day. As

mentioned in previous reports, the decreased growth correlates with the decreased food consumption, but the decreased food consumption, deaths and pathological lesions are inherent to the toxicant present.

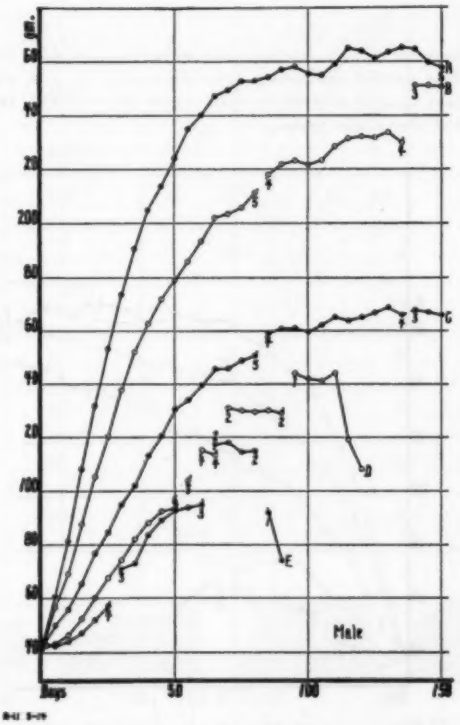


Fig. 1 Average growth curves of males obtaining: group A, toxic corn lab. no. 388, 0.0 per cent of diet; group B, toxic corn lab. no. 388, 17.5 per cent of diet; group C, toxic corn lab. no. 388, 35.0 per cent of diet; group D, toxic corn lab. no. 388, 52.5 per cent of diet; group E, toxic corn lab. no. 388, 70.0 per cent of diet (the sub-numerals indicate the number of animals averaged).

### *The preliminary feeding of toxic foodstuffs followed by control foodstuffs*

Forty rats were divided into four groups and placed in individual drawer cages (Franke and Franke, '34) and fed as follows:

Group A—30 days on diet containing toxic wheat lab. no. 459, then control diet.
Group B—20 days on diet containing toxic wheat lab. no. 459, then control diet.
Group C—10 days on diet containing toxic wheat lab. no. 459, then control diet.
Group D—Control diet entire period.

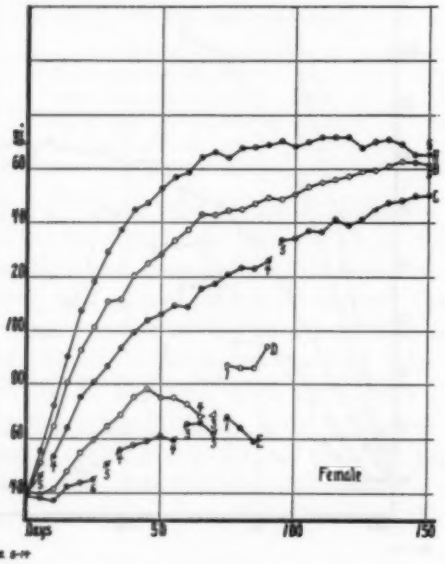


Fig. 2 Average growth curves of females (same as above).

The composition of the diet was the same as for the first series, except that wheat was used in place of corn. The growth curves and food intake curves of these rats are given in figure 3. In group A one rat died before the thirtieth day of experimentation when they were changed to the diet containing control wheat. Even though the others responded to the change of diets, seven had been affected so severely that they died by the one hundredth day of experimentation.

The other two were killed on the one hundred and seventy-fifth day.

In group B the same immediate response to change of diet is noted, but here again the damage to the system had been

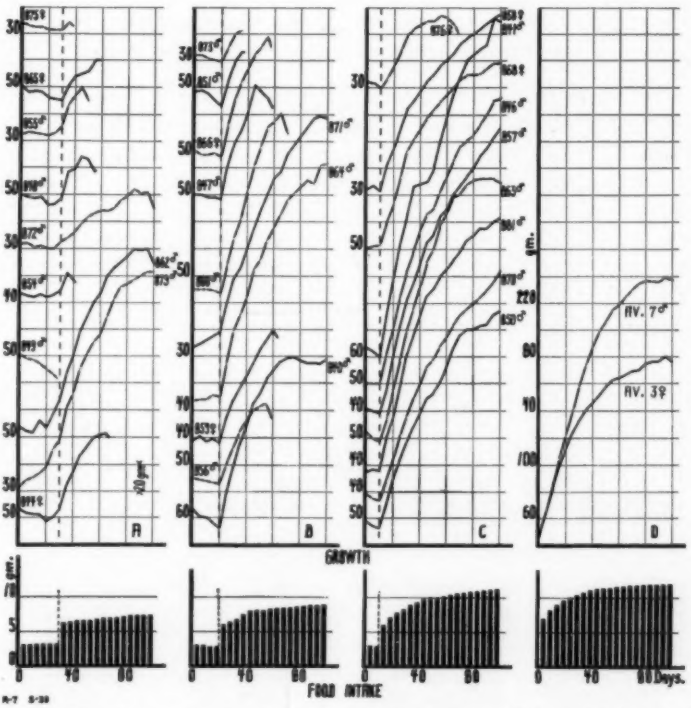


Fig. 3 Growth curves of rats fed: first 30 days on diet containing toxic wheat, then on diet containing control wheat; first 20 days on diet containing toxic wheat, then on diet containing control wheat; first 10 days on diet containing toxic wheat, then on diet containing control wheat. Control group. Food intake of rats obtaining toxic wheat first 30 days, first 20 days, first 10 days, and control.

so severe that it caused the death of seven out of ten rats by the seventieth day. In group C only one rat died (on sixty-eighth day). The others were killed on the one hundred and seventy-fifth day. The differences in food intakes in these

groups are very interesting. The sharp increases noted each time the rats are changed to the control diet are especially interesting.

It was of interest that four litter mates (one in each group) were living on the one hundred and seventy-fifth day. When these were killed, the weights of the liver, spleen, testes and epididymides were so different that they are given:

| RAT NO. | GROUP | WEIGHT IN GRAMS | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Body | | Food consumed | Liver | Spleen | Testes | Epididymides |
| | | Start | End | | | | | |
| 862 | A | 57 | 226 | 1516 | 5.90 (2.61) | 1.00 (0.44) | 1.00 (0.44) | 0.60 (0.27) |
| 864 | B | 47 | 279 | 1858 | 7.35 (2.63) | 0.80 (0.29) | 1.10 (0.39) | 0.65 (0.23) |
| 863 | C | 54 | 284 | 1859 | 7.60 (2.68) | 0.80 (0.28) | 1.30 (0.46) | 0.70 (0.25) |
| 861 | D | 48 | 274 | 1941 | 9.00 (3.28) | 0.55 (0.20) | 3.05 (1.11) | 1.15 (0.42) |

Figures in parentheses are based on 100 gm. body weight.

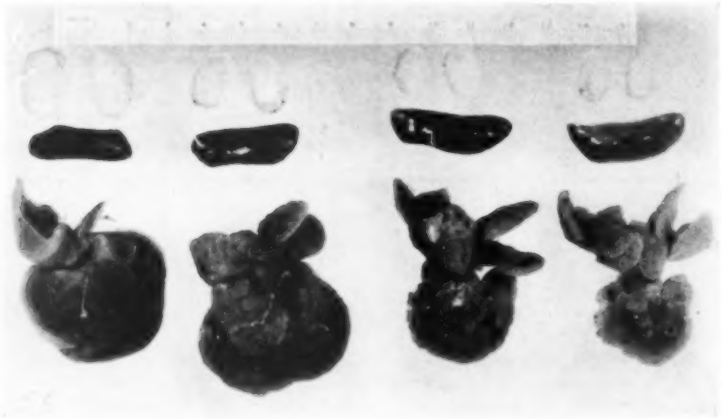

Fig. A Testes, spleen and liver from male litter mates: Rat no. 861, control; 865, 10 days; 864, 20 days; 862, 30 days on diet containing toxic corn.

It will be noted that the body weights are fairly uniform. Therefore, the organ weights are not abnormal because of differences in growth, but are due to a pathological condition caused by the toxic foodstuff. The testes and epididymides were not as firm as those from control rats.

The gross appearance of the livers, spleens, and testes are shown in figure A. In the post mortems of the other rats similar conditions were noted, but in varying degrees.

## DISCUSSION

From the first series in which the question of the effect of dilution of toxic foodstuffs was studied, the conclusion might be drawn that all livestock in the affected area is subnormal to a greater or lesser degree. It has been shown that the toxic constituent is not constant in the plant materials grown in any one area (Franke, '34); just as the soil varies in its chemical composition, and weather conditions vary, so also the composition of plants grown thereon may vary. The fact must not be overlooked that subnormal animals may exist without being noticed.

In the second series when the rats were fed diets containing toxic wheat for 30-, 20-, and 10-day periods followed by control diet, it must be recognized that the restriction of food intake was entirely voluntary. Stewart ('16), by underfeeding from 3 to 10 weeks, and following this by generous feeding, came to the conclusion that there was "a marked tendency of the body as a whole in young rats to recover its weight after a period of maintenance is likewise characteristic of the various organs and parts." Jackson and Stewart ('18) concluded that the suppression of growth in young rats affects their subsequent capacity to grow according to the age of the rats and the length of severity of the underfeeding. That is to say, the effect is inversely proportional to the age of the animal and directly proportional to the length of time underfed.

Jackson ('25) later states, "It is well known that recovery from inanition is generally possible if adequate nutrient is provided before the extreme stage is reached," but he calls attention to the fact that there is disagreement as to the possibility of a permanent stunting in the later growth of the body. He also takes up the question of the cause of death from inanition, either total or partial, and concludes that the

immediate cause may vary according to circumstances. Death may be a direct result of inanition or an indirect result caused by toxins in the circulation, or it may be due to a lowered resistance to infection known to be a result of various types of inanition, either total or partial. He states, "On the other hand, since most diseases, especially the chronic disorders, interfere more or less with the process of nutrition, inanition is usually present as a complication."

It is felt that this statement covers the work described in this series of papers in that the usual question of the lack of essential proteins, salts, vitamins and water probably does not enter into this work. However, many of the pathological symptoms generally connected with these can be observed.

When one considers that the animals do regain body weights by being fed control wheat, a concurrence with these statements of Stewart and Jackson will be found, but when the organs, especially the liver, spleen and kidneys, and in many cases the reproductive organs are studied, it is found that profound pathological changes have taken place so that the normal development of these organs probably does not take place.

## CONCLUSIONS

A dilution of toxic grain so that only 17.5 per cent is contained in the diet showed definite depressed growth rates and also caused deaths.

Concentrations of 35 per cent and more caused still greater depressions of growth, and a greater number of deaths.

The effect of feeding diets containing toxic wheat for 30-, 20-, and 10-day periods, followed by control diet, was studied. Pathological effects resulted even for the 10-day period on toxic diet. Although normal growth was resumed when the rats were changed to the control diet, the damage to the organs was never repaired.

The pathological changes decreased as the intake of toxic food decreased.

## LITERATURE CITED

BURR, G. O., AND M. M. BURR 1929 A new deficiency disease produced by the rigid exclusion of fat from the diet. J. Biol. Chem., vol. 82, p. 345.

FRANKE, KURT W. 1934 A new toxicant occurring naturally in certain samples of plant foodstuffs. I. Results obtained in preliminary feeding trials. J. Nutrition, vol. 8, p. 597.

FRANKE, KURT W., AND W. R. FRANKE 1934 A metabolism cage for rats. J. Lab. and Clin. Med., vol. 19, p. 669.

JACKSON, C. M. 1925 The effects of inanition and malnutrition upon growth and structure. P. Blakiston's Son and Company, Philadelphia.

JACKSON, C. M., AND C. A. STEWART 1918 The effects of underfeeding and refeeding upon the growth of the various systems and organs of the body. Minn. Med., Nov.

STEWART, CHESTER A. 1916 Growth of the body and of the various organs of young albino rats after inanition for various periods. Biol. Bull., vol. 31, pp. 16–51.